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CHEMOTHERAPY AND BIOCHEMISTRY OF LEISHMANIA

ANNUAL REPORT

LINDA L. NOLAN, Ph.D.

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A comparison of the enzymes of the pathogenic protozoa to those of man is of fundamental importance to the search for much needed chemotherapeutic agents. Nucleic acid metabolism in trypanosomatids is unique in several ways: (1) they lack the ability to synthesize purines do novo, depending entirely on the salvage pathway for their supply of purine nucleotides; (2) many of the enzymes involved in nucleic acid biosynthesis either have unusual substrate specificities or unusual subcellular localizations; (3) a large proportion of the DNA which is produced is incorporated into a unique organelle known as the kineto-		

plast; and (4) the DNA polymerase isolated from these organisms demonstrates major differences from its mammalian counterpart.

There is very little information concerning the DNA and RNA polymerases of Leishmania spp.

Our aim has been the isolation and characterization of the DNA and RNA polymerases of Leishmania mexicana and search in vivo and in vitro for inhibitors of these enzymes for chemotherapeutic exploitation.

Sinefungin has been found to be a potent antiparasitic agent at levels which are non toxic to mammalian cells. Our laboratory has found that it drastically affects DNA synthesis in Leishmania spp. We are currently investigating its exact mode of action, to aid in rational drug development.

We have continued our studies on the mode of action of Formycin B, an anti-leishmanial purine analogue, and have shown that it is converted to Formycin A and preferentially incorporated into mRNA as opposed to tRNA and rRNA.

to provide anti-leishmanial agents; (17).

TABLE OF CONTENTS

Report Documentation Page	2-3
Resume of Progress	5
Isolation and Characterization of DNA Polymerase	6-20
RNA Polymerase Studies	21-38
Incorporation of Formycin B into RNA	38-42
Inhibition of Leishmanial DNA Synthesis by Sinefungin	43-51
Culture Methods	52
Inhibition by Purine Analogues	54-55
References	56-58



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RESUME OF PROGRESS

During the last year we have:

- Continued our isolation and characterization of the DNA polymerases of Leishmania mexicana WR 227.
- Continued our isolation and characterization of RNA polymerase of L. mexicana WR 227.
- Investigated the mode of action of sinefungin which has been shown previously to be extremely leishmanicidal in a promastigote test system.
- We have continued our studies on the mode of action of Formycin B, and have found that it is converted to Formycin A and preferentially incorporated into mRNA as opposed to tRNA and rRNA.

We have combined several promising antileishmanicil compounds, and have shown that they act synergistically in a promastigote test system.

A. ISOLATION AND PARTIAL CHARACTERIZATION OF AN α -LIKE DNA POLYMERASE FROM
LEISHMANIA MEXICANA

SUMMARY. This report describes for the first time the isolation and characterization of the predominant DNA polymerase from the genus Leishmania which are parasitic flagellated protozoa. Like mammalian DNA polymerase α , the leishmanial DNA polymerase is of large molecular weight, is sensitive to N-ethylmaleimide, and is inhibited by high ionic strength. Unlike mammalian DNA polymerase α , but similar to the predominate DNA polymerase isolated from the related organisms Trypanosoma cruzi and Crithidia fasciculata, the leishmanial DNA polymerase is resistant to inhibition by aphidicolin, a potent inhibitor of DNA replication in mammalian cells and of DNA polymerase α . The DNA polymerase α -like was purified over 4,000 fold and properties such as pH optimum, salt sensitivity, template requirements and response to DNA polymerase inhibitors were determined. A DNA polymerase β -like could not be detected during the isolation procedures.

Leishmania species are unicellular hemoflagellates which infect millions of persons in the tropical and semitropical regions of the world. Leishmaniasis is initiated when sandflies inject the extracellular promastigote form of the parasite into the skin. The promastigotes are phagocytized by macrophages and transform into the amastigote stage which is responsible for the clinical disease.

Three main classes of DNA polymerase have been defined in mammalian cells, DNA polymerase α , β and γ (1). DNA polymerase γ is now believed to be the mitochondrial DNA polymerase (2). DNA polymerase β , the lower molecular weight and comparatively N-ethylmaleimide-resistant species of DNA-dependent DNA polymerase, has not been found in plants (3-5). Recently, a β -like DNA polymerase was detected in Trypanosoma brucei and Crithidia fasciculata (6,7), but another study failed to detect a β -like enzyme in T. brucei (8).

Little is known about the DNA polymerase of parasitic protozoa. A DNA polymerase α -like was reported in T. brucei (8), T. cruzi (9), and C.

fasciculata (7). This is the first report describing the purification and characterization of the predominate DNA polymerase in Leishmania.

This report on the leishmanial DNA polymerase and the report by others describing DNA polymerase from other parasitic protozoa show that although similarities exist between parasitic and mammalian DNA polymerases, there are sufficient differences to encourage the view that these enzymes may be exploited for chemotherapy.

MATERIALS AND METHODS

[³H]TP (45 Ci mmole⁻¹) was purchased from Amersham. Heparin-Sepharose CL-6B, cellulose phosphate, and native and denatured DNA cellulose were obtained from Pharmacia Fine Chemicals. 2-Arylamino-purine deoxyribonucleoside 5'-triphosphates were obtained from Dr. George E. Wright, University of Massachusetts Medical School, Worcester, MA. Aphidicolin was supplied by A.H. Todd of Imperial Chemical Industries, England. All other chemicals were of the highest purity and were obtained from Sigma Chemical Co., except soybean trypsin inhibitor, aprotinin and leupeptin which were crude grade.

Cell Culture Conditions: Promastigotes of Walter Reed Strain #227 were used in these experiments. This strain has been previously identified as Leishmania mexicana amazonensis (J. Decker-Jackson and P. Jackson, personal communication), and was obtained from the Leishmania Section of the Walter Reed Army Institute of Research. Promastigotes were grown in Brain Heart Infusion Medium (BHI) containing 37g BHI (Difco) L⁻¹ water, 10% heat inactivated serum and 26 µg hemin ml⁻¹. Cells were grown at 26° in 2000 ml wide Fernbach flasks containing 250 ml of BHI. Cells were harvested after 4 days during the exponential growth phase. The cell density was 4-6 x 10⁷ cells ml⁻¹.

Purification of leishmanial DNA polymerase: Cells (1 liter) were harvested in 250 ml centrifuge bottles and centrifuged at 12,000 rpm for 10 min. The cells were washed twice in buffer containing 50 mM Tris-HCl (pH 7.5). The cell pellet (usually 4-6g wet wt.) was suspended in lysis buffer (8-12 ml) containing 10 mM Tris-HCl (pH 7.5), 20% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1.0 M HCl and 0.3% Triton X-100. This cell suspension was supplemented (1:100) with the following stock solution of protease inhibitors: soybean trypsin inhibitor (4.8 mg ml⁻¹), aprotinin (4.8 mg ml⁻¹) and leupeptin (2 mg ml⁻¹). Isolated cells were homogenized at 4° with a Teflon homogenizer or sonicated 5 times for 10 S with 2 min. cooling intervals. This was carried out with a Braun-Sonic 2000 at 4°. The suspension was centrifuged at 15,000 rpm for 1 hr. at 4°. This supernatant fluid was subjected to protamine sulfate treatment, dialysis and column chromatography. DNA was removed by adding sufficient 2% protamine sulfate to the crude enzyme to result in a 1:10 dilution. The precipitate from the protamine sulfate step was removed by centrifugation at 15,000 rpm for 15 min at 4°. The supernatant was dialyzed in 2 L of standard buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM dithioerythritol, 1 mM EDTA, and 20% glycerol. The precipitate that formed after dialysis was removed by centrifugation at 15,000 rpm for 20 min at 4°.

Chromatography: The supernatant was applied to a Heparin-Sepharose column (8.5 cm x 1.3 cm) equilibrated with 0.1 M KCl in standard buffer. The column

was washed with this buffer until the absorbance at 280 nm was less than 0.1. The DNA polymerase was then eluted with 0.5 M KCl in standard buffer. Active fractions were pooled and protease inhibitors were added as described. The pooled fractions were dialyzed overnight in 2 L of standard buffer containing 0.1 M KCl. After dialysis more protease inhibitors were added as described, and the pooled fractions were applied to a cellulose-phosphate column (7.0 cm x 1.3 cm) equilibrated with 0.1 M KCl in standard buffer. The column was washed with the same buffer until the absorbance at 280 nm was less than 0.1. The DNA polymerase was then eluted in 0.35 M KCl in standard buffer. The active fractions were pooled and protease inhibitors were added. The fractions were dialyzed in 2 L of standard buffer overnight. The pooled dialyzed fractions were applied to either a native or denatured DNA cellulose column (13.0 cm x 1.0 cm) equilibrated with standard buffer. The column was washed first with 15 ml of standard buffer, and 15 ml step gradients were applied consisting of 0.1 M KCl and 0.25 M KCl in the same buffer.

Protein assays: Protein concentrations were either determined by the dye-binding method (Bio-Rad Labs) or by a modified method. The modified method was performed in 96 well microplates by adding 80 μ l of Bio-Rad dye and 20 μ l of a column fraction. The plate was then read in a Dynetech 580 microplate reader at 575 nm.

Denatured DNA: Calf thymus DNA (Sigma, 0.5 mg/ml) was heated in a water bath at 90°C for 5 min and then quickly chilled on ice.

Molecular weight determination: The DNA polymerase isolated from DNA cellulose chromatography was subjected to gel filtration on a Sephacryl S-300 column (72 cm x 1.8 cm) equilibrated with 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, and 100 mM KCl. Fractions of 1.5 ml were collected. Molecular weight values for standard proteins were plotted against their elution volumes. Standard proteins used for the molecular weight determinations were: thyroglobin, apoferritin, β -amylase, alcohol dehydrogenase, and bovine serum albumin.

DNA polymerase assay: Enzymes were assayed for activated DNA dependent activity in a reaction mixture containing 10 mM Tris-HCl (pH 7.5), 41.6 μ M each of dATP, dCTP, dGTP and [methyl- 3 H]dTTP at 200-300 cpm pmol $^{-1}$, 5 mM MgCl $_2$, 100 μ g ml $^{-1}$ bovine serum albumin and 50 μ g ml $^{-1}$ activated calf thymus DNA. Assays were for 30 min at 30°. Compounds tested for inhibition were preincubated 15 min at 30° with the enzyme to be tested. Assays were routinely carried out in a final volume of 50 μ l. Reactions were terminated by pipetting 10 μ l of a solution containing 2.5% SDS and 0.15 M sodium pyrophosphate (10). The reaction mixture was then pipetted onto DEAE-cellulose discs (Whatman DE81) followed by immersion in 0.5 M Na $_2$ HPO $_4$. The filters were washed 5 times for 5 min each in this buffer, twice in distilled H $_2$ O, twice in 95% EtOH, once in ether, and air-dried. The discs were counted in Fisher Scinti Verse II in a Beckman scintillation counter.

One unit of activity is defined as the incorporation of 1 pmole of dTTP into DNA in 30 min under standard assay conditions.

RESULTS AND DISCUSSION

Crude extracts of L. mexicana contain DNA polymerase activity. Only one major DNA polymerase was purified from total extracts. When protease inhibitors were used as described only one major DNA polymerase was obtained. If

protease inhibitors were not used during all phases of purification a second DNA polymerase peak occurred, but this peak exhibited the same α -like properties as the major peak. The second peak may be due to proteolysis. Figure 1 shows the two peaks obtained during DNA cellulose chromatography when protease inhibitors were omitted during the extraction procedure. The molecular weight as determined by gel filtration on Sephacryl S-300 was 130,000. The DNA polymerase was purified 4,416 fold using homogenation, protamine sulfate treatment to remove nucleic acids, and chromatography on heparin-sepharose, cellulose phosphate and DNA cellulose (Table 1). The most efficient step in the isolation procedure appears to be chromatography on DNA cellulose after chromatography on cellulose phosphate. The specific activity increased dramatically and 92% of the protein from the previous step was removed. During all phases of purification the enzymatic activity was sensitive to N-ethylmaleimide and KCl. N-ethylmaleimide (9 mM) resulted in 94-100% inhibition, and KCl (200 mM) 77-100% inhibition of the enzyme during varying phases of the purification. Aphidicolin, an inhibitor of DNA replication in mammalian cells and of DNA polymerase α , had no effect on DNA polymerase activity after chromatography. Concentrations as high as 500 μ M were used in the presence and absence of dCTP. As shown in Fig. 2, aphidicolin is a potent growth inhibitor of L. mexicana. Failure of aphidicolin to inhibit DNA polymerase in vitro may be due to the presence of a tight binding protein on the enzyme protecting it from inhibition. Foster et al. reported that resistance of adenoviral DNA replication to aphidicolin is dependent on the 72-kilodalton DNA-binding protein (11). Also, failure of aphidicolin to inhibit DNA polymerase in vitro may be due to an entirely different mode of action of this compound in parasitic protozoa. The DNA polymerases isolated from the parasitic protozoa, T. cruzi, and C. fasciculata, have also been shown to be resistant to inhibition by aphidicolin (7,9). Recently, aphidicolin has been found to inhibit conversion of lanosterol

Table 1.
Purification Scheme for DNA Polymerase

Purification Step	Total Volume (ml)	Total Protein (mg)	Units* (ml) ⁻¹	Total Activity (units)	Specific Activity (units mg ⁻¹)	Purification (-fold)
Lysed cells	40.8	1448.4	6.70	273.18	0.189	
Crude extract (15,000 rpm, 1 hr)	38.5	1068.38	7.7	296.45	0.277	1.47
Protamine Sulfate Supernatant	37.5	441.0	15.29	573.36	1.3	6.88
Heparin-Sepharose	14.5	41.62	119.77	1736.67	41.73	220.79
Cellulose Phosphate	10.2	4.38	26.58	271.14	61.82	327
DNA Cellulose	3.1	0.108	29.08	90.15	834.72	4416.51

*One unit of activity is defined as the incorporation of 1 pmole of dTMP into DNA in 30 min under standard assay conditions. Assay conditions are described in Methods.

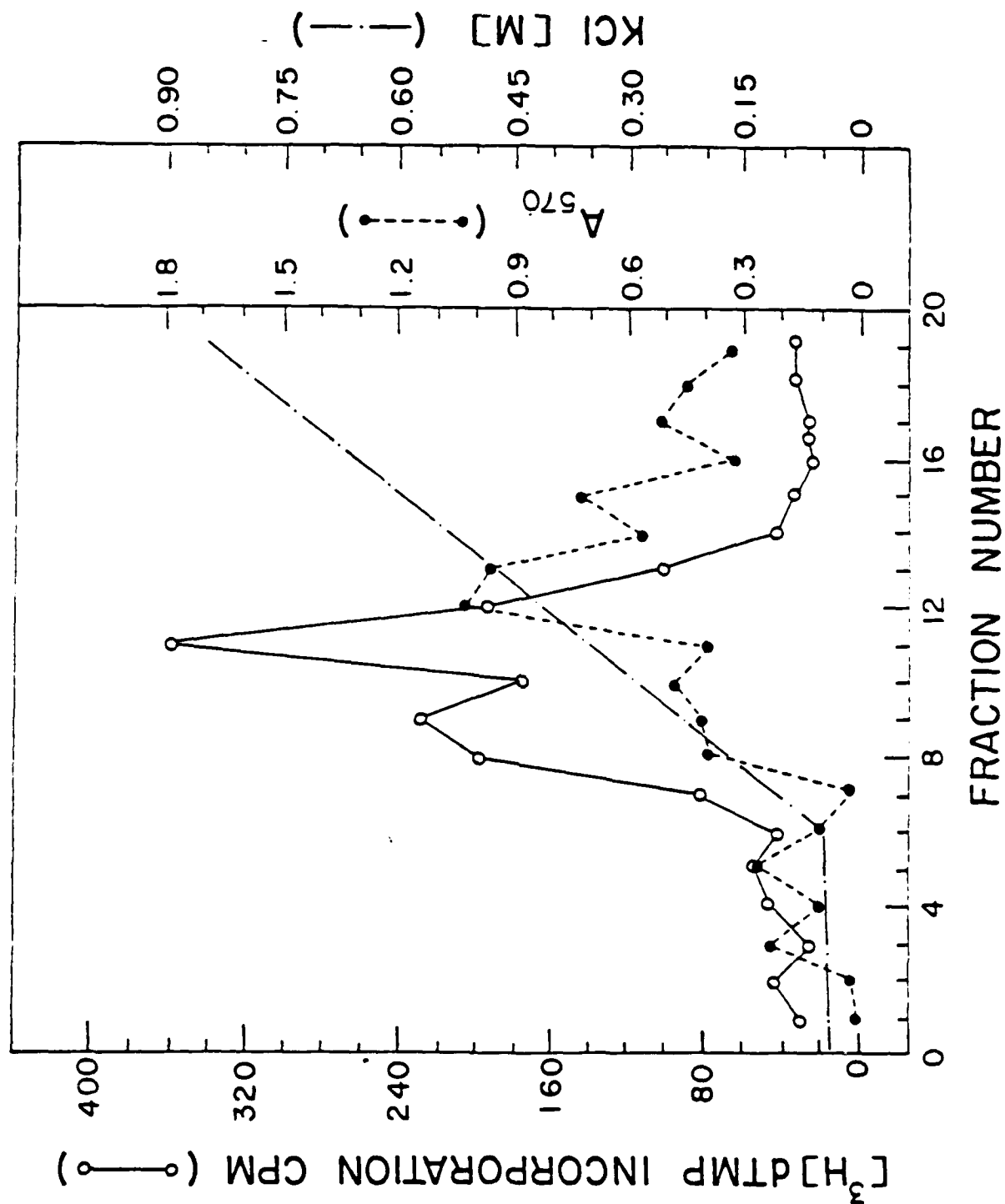


Fig. 1. Chromatography of *L. mexicana* DNA polymerase on native DNA cellulose without the addition of protease inhibitors as described in Methods. The enzymes were eluted with a KCl gradient (0-0.90 M). Two ml fractions were collected. Both peaks of enzymatic activity exhibited the same sensitivity to salt concentration, N-ethylmaleimide, and were resistant to inhibition to aphidicolin.

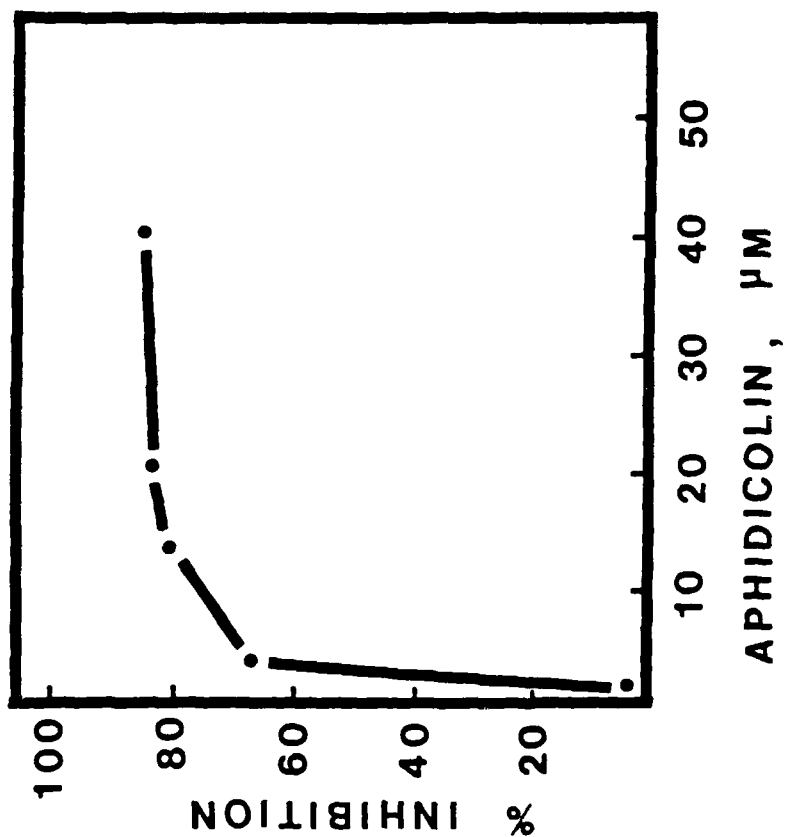


Fig. 2. Growth inhibition of *L. mexicana* by aphidicolin. Cells were grown in 5 ml of the defined medium of Steiger and Black (20) without adenosine and supplemented with 5% heat inactivated serum. Growth was monitored by turbidity measured at 660 nm at 72 hrs.

to C-27 sterols in mouse L cells (12). We are currently investigating the action of aphidicolin on sterol metabolism in leishmania to determine if its in vivo inhibitory action is due to inhibition of sterol biosynthesis. Figure 3 shows the sensitivity of the enzyme to KCl, 200 mM producing 95% inhibition of activity. As shown in Table 2, and Figs. 4 and 6, the leishmanial DNA polymerase is extremely sensitive to ethidium bromide, berenil and arachidonic acid. The inhibition of DNA synthesis by ethidium bromide and berenil could involve interaction of the compounds with the DNA template. Ethidium bromide has been used as a chemotherapeutic agent for the treatment of trypanosomiasis (13). Phosphonoacetic acid, which has been reported to be a specific inhibitor for DNA polymerase induced by infection by Herpes virus (14), inhibited only slightly at 100 μ M (Table 2) BuPdGTP and BuAdATP which have been found to inhibit mammalian DNA polymerase α from a variety of sources (15), was much less active against the leishmanial DNA polymerase.

Arachidonic acid, a precursor to the prostaglandins and modulator of the immune response (16-18) was found to be both a potent growth inhibitor of promastigotes (50% inhibition at 0.5 μ M, data not shown) and a potent DNA polymerase inhibitor of L. mexicana (Fig. 6). To our knowledge this is the first report showing that arachidonic acid inhibits any DNA polymerase. It has been reported that inhibitors blocking arachidonic acid metabolism during infection help restore immune function (19). Perhaps release of arachidonic acid by the action of phospholipase A during infection is an attempt by the host to kill its invader. If the invader then metabolizes the released arachidonic acid to prostaglandins by its own enzymes and suppresses the immune response (as leishmania do [16]), it has successfully evaded its host. Several prostaglandins and linoleic acid have been found to have no effect on the leishmanial DNA polymerase (data not shown).

The nucleotide analogs ddTTP and araCTP were found to be inhibitory (Fig. 5, Table 2), with ddTTP showing the greatest inhibition. A similar response

Table 2. In vitro inhibition of L. mexicana DNA polymerase. Incubation was carried out for 30 min. with activated DNA template as described in Methods. Inhibitors were preincubated at 30° for 15 min. with the enzyme. When using purine analogs, the natural purine concentration was varied from 8-42 μ M. The value given for % inhibition when using purine analogs was the maximum inhibition obtainable under the experimental conditions.

Inhibitor	Concentration	% Inhibition
N-ethyl maleimide	5-10 mM	94
Berenil	3 μ M	50
	100 μ M	90
Ethidium bromide	50 μ M	78
Arachidonic acid	79 μ M	50
	100 μ M	98
Phosphymycin	100 μ M	50
Phosphonacetic acid	50 μ M	20
ddTTP	100 μ M	59
BuPdGTP	320 μ M	46
AraCTP	100-200 μ M	20
KCl	60 mM	50
Aphidicolin	30-300 μ M	None
Spermine	30 μ M	12% Stimulation
Linoleic	10 μ M	None
BuAdATP	250 μ M	None

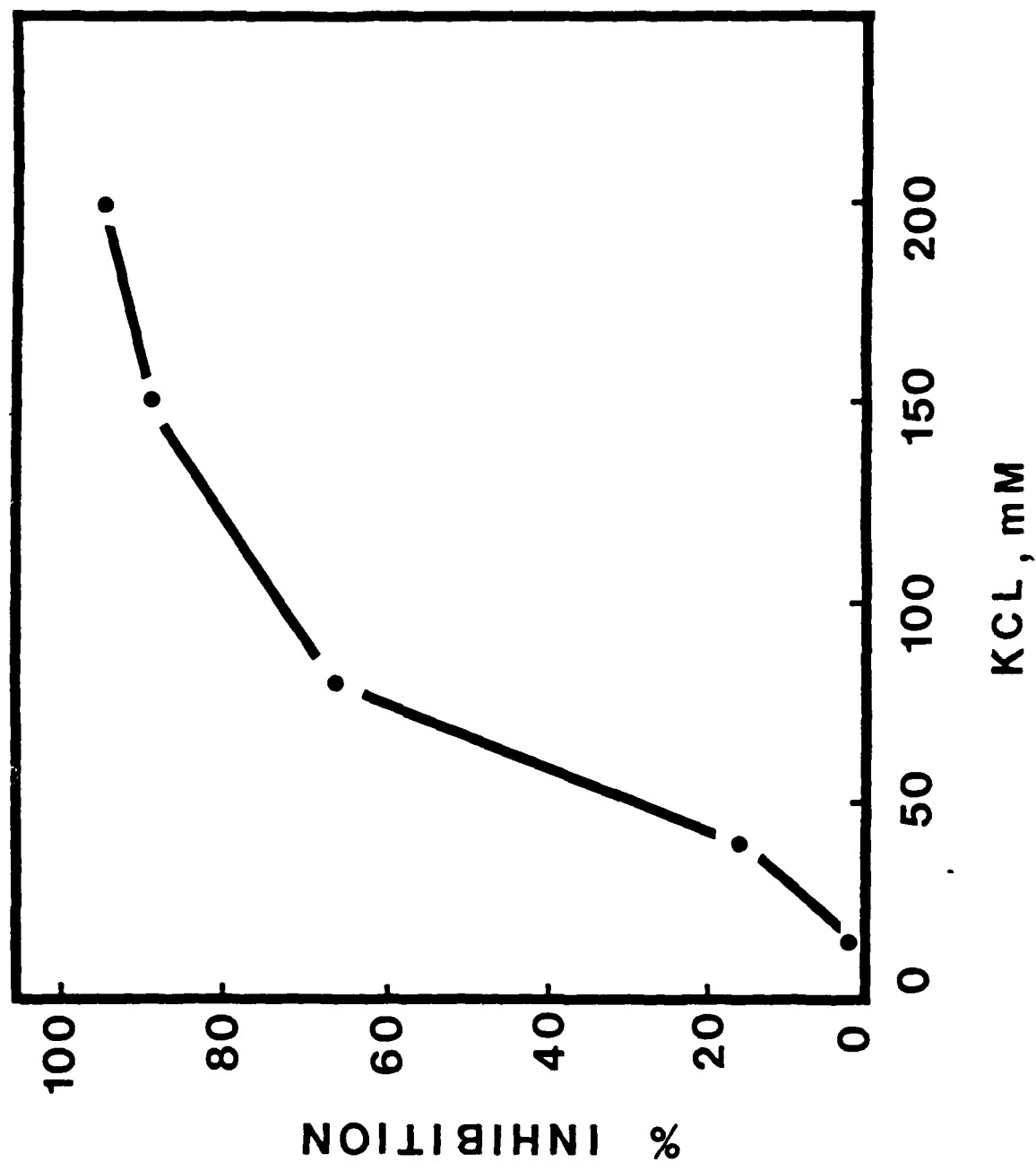


Fig. 3. Sensitivity of *L. mexicana* DNA polymerase to KCl concentration. The enzyme tested chromatographed as a single peak from a denatured DNA cellulose column.

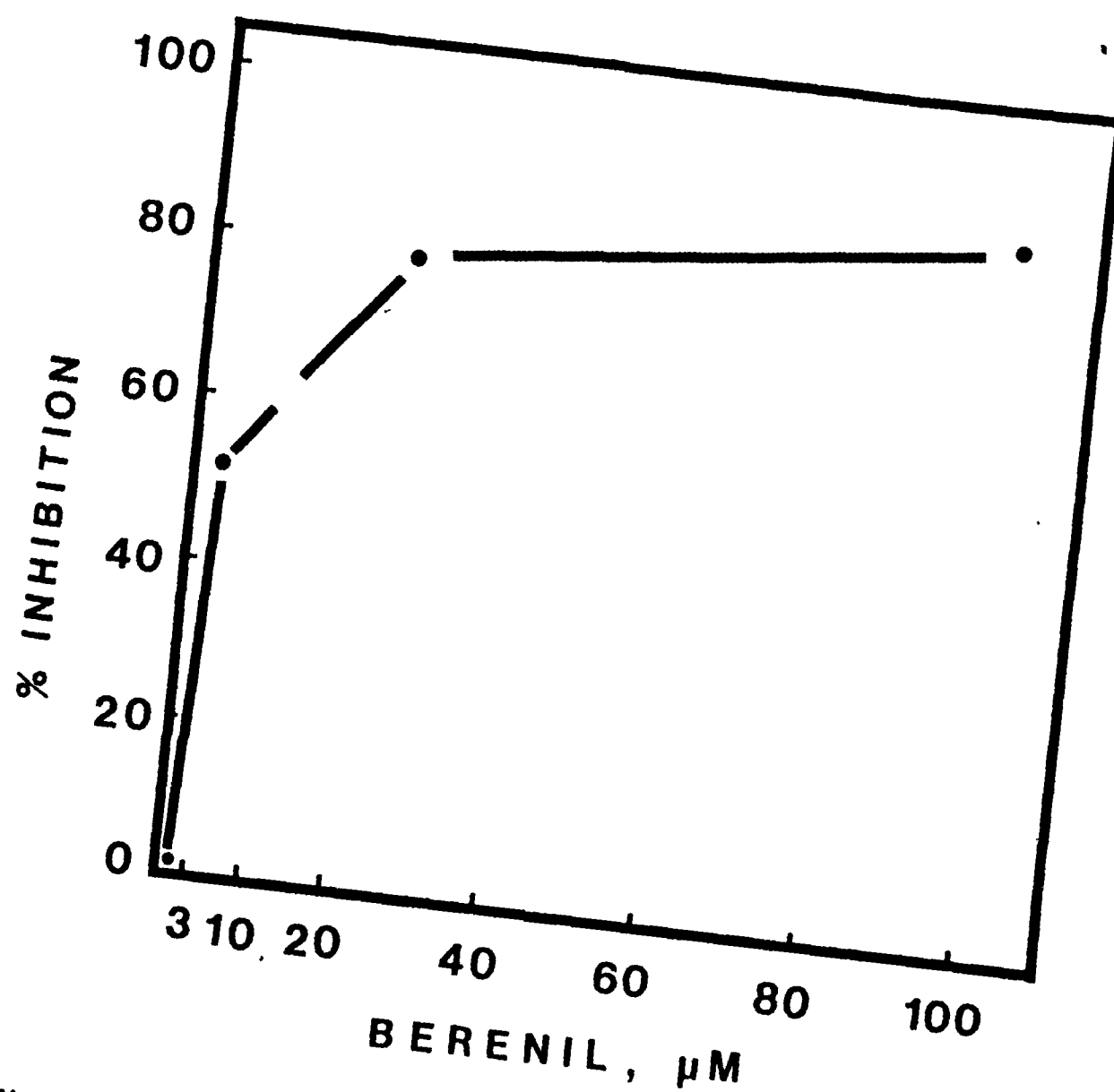


Fig. 4. Inhibition of *L. mexicana* DNA polymerase by berenil (diaminazine acetate). Activated calf thymus DNA (50 $\mu\text{g}/\text{ml}$) was used as a template. Assay was performed as described in Methods.

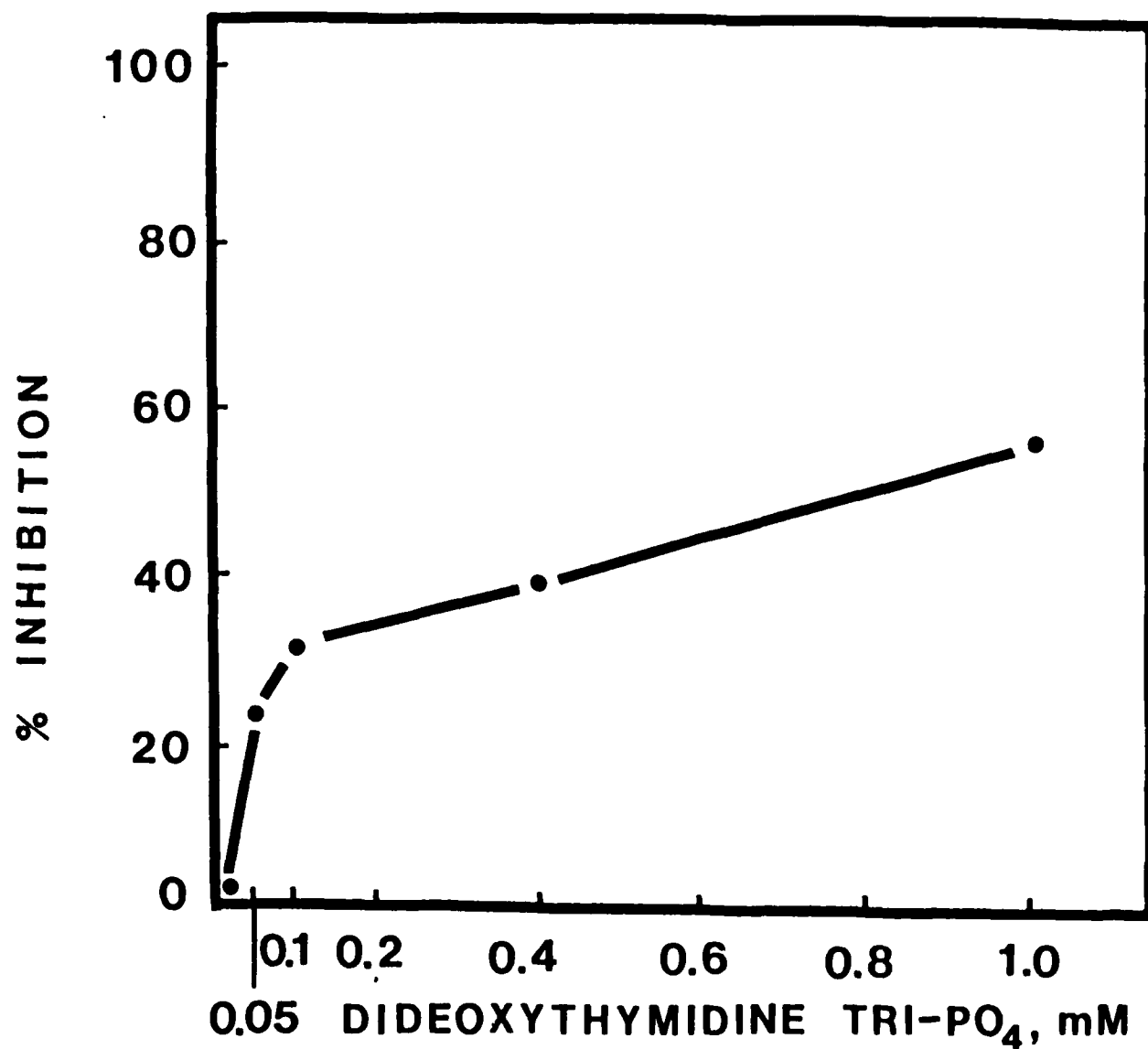


Fig. 5. Response of *L. mexicana* DNA polymerase to the purine analog, dideoxythymidine triphosphate. Enzyme source eluted as a single peak from a denatured DNA cellulose column. Assay conditions are described in Methods. Each assay contained 0.2 units of DNA polymerase.

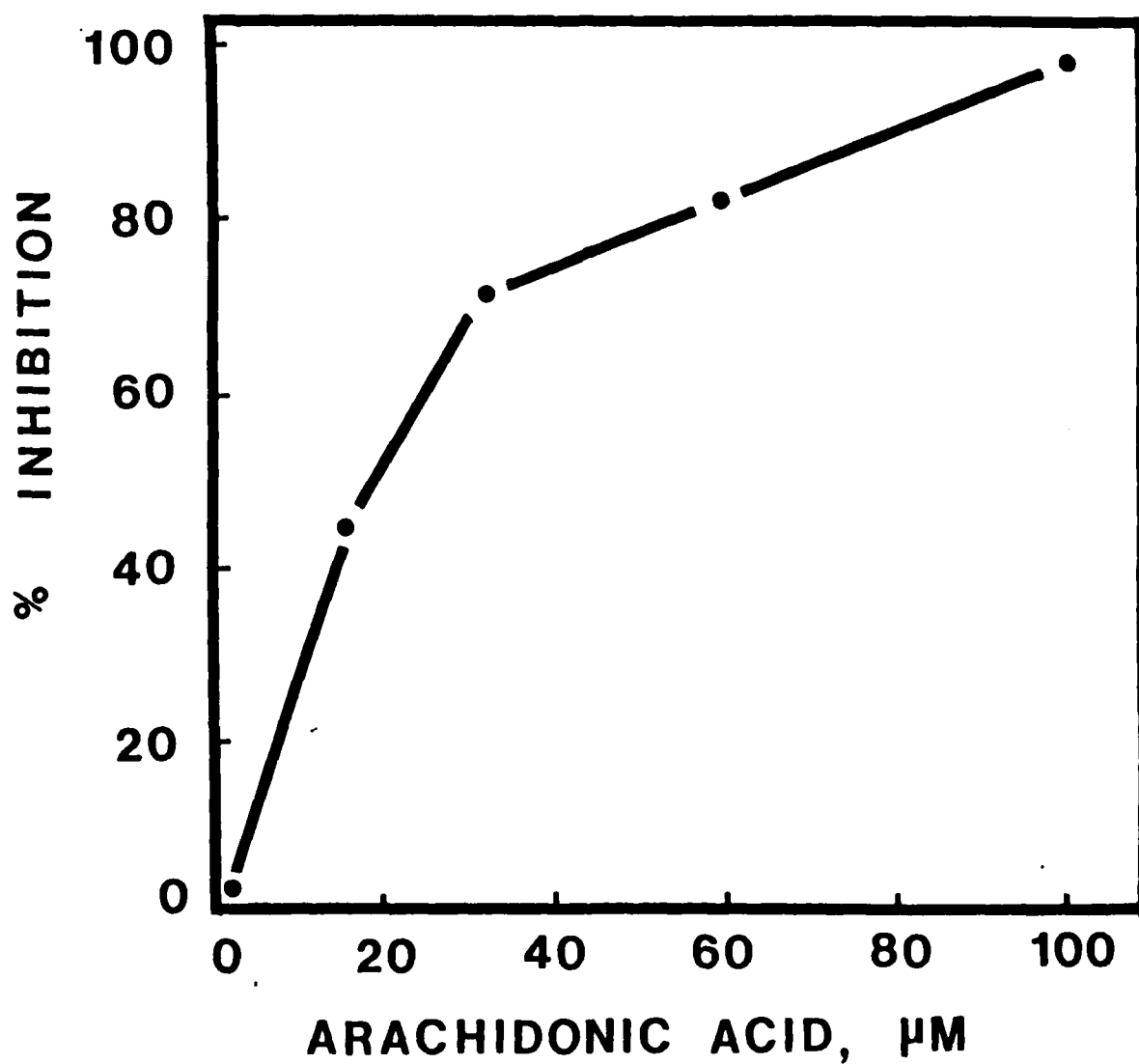


Fig. 6. Inhibition of *L. mexicana* DNA polymerase by arachidonic acid. Assay conditions are described in Methods. Each assay contained 0.2 units of DNA polymerase which was isolated as a single peak from a denatured DNA cellulose column as described in Methods.

was observed with the DNA polymerase isolated from T. cruzi (9). The DNA polymerase from L. mexicana is extremely sensitive to activated DNA template concentrations. As shown in Fig. 7, $50 \mu\text{g ml}^{-1}$ activated DNA gave optimum activity, and increasing the concentration caused inhibition of the enzyme.

This is the first report describing the purification and partial characterization of the predominate DNA polymerase from the genus Leishmania. It appears from our findings that this enzyme is very similar to the major DNA polymerase isolated from related parasitic protozoa (6-9). It is similar in characteristics to mammalian DNA polymerase α regarding sensitivity to salt and N-ethylmaleimide, but is strikingly different in being resistant to inhibition by aphidicolin and 2-arylaminopurine deoxyribonucleoside 5' triphosphates. Recent reports have demonstrated that the DNA polymerases of the related pathogenic protozoa, T. brucei and C. fasciculata, are immunologically distinct and have suggested that structural differences between the parasite and the host enzymes could be exploited for the development of agents to combat parasitic diseases (6-8). The evidence emerging concerning the DNA polymerase(s) of these lower eukaryotes suggests that they represent something of an intermediate between that of prokaryotes and higher eukaryotes.

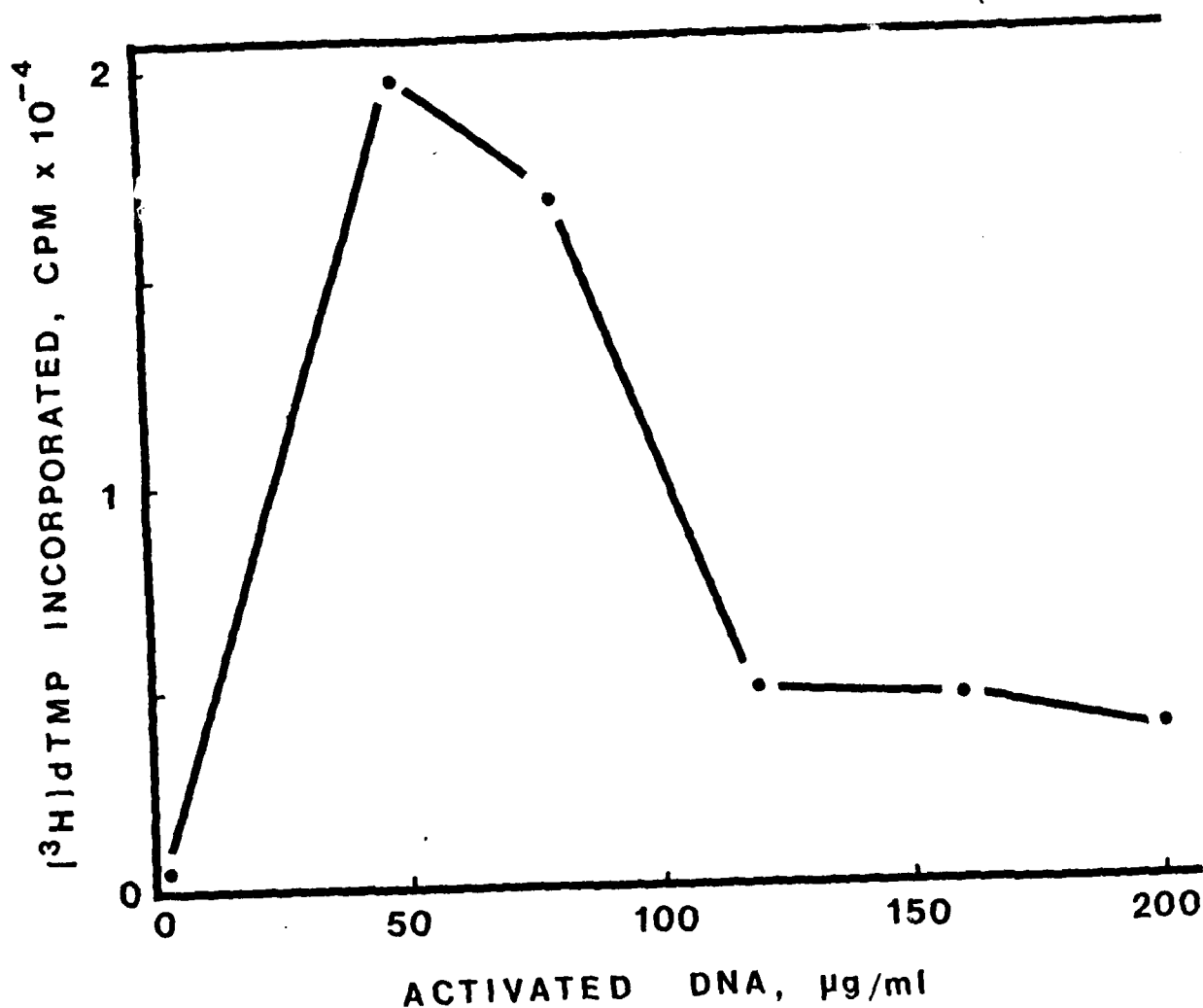


Fig. 7. Response of *L. mexicana* DNA polymerase to varying concentrations of activated DNA template. Maximum activity was obtained with 50 $\mu\text{g/ml}$ when using the assay as described in Methods. Concentrations higher than 50 $\mu\text{g/ml}$ decreased activity significantly. When native DNA was used at 50 $\mu\text{g/ml}$ only one-third of the activity was obtained when compared to denatured DNA (data not shown). Each assay contained 0.25 units of DNA polymerase.

RNA POLYMERASE STUDIES

SUMMARY

A DNA-dependent RNA polymerase has been isolated and characterized from the parasitic flagellated protozoan, Leishmania mexicana. The initial stages of purification utilize high ionic strength extraction and protamine sulfate treatment. The enzyme was further purified by differential elution on Heparin-Sepharose, DEAE-Sephadex, and Carboxymethyl-Sephadex chromatography (Table 3). Analysis of the chromatographically purified RNA polymerase on nondenaturing gels revealed two electrophoretic forms. The enzyme isolated has characteristics of true DNA-dependent RNA polymerase since it requires DNA and all four nucleoside triphosphates for the synthesis of RNAase-sensitive products. Analysis of ammonium sulfate and metal ion optima, as well as relative activities of the enzyme with Mn^{2+} versus Mg^{2+} are similar to those reported for other RNA polymerase III in eukaryotes.

Formycin A triphosphate was found to be a competitive substrate for this enzyme, and cordycepin triphosphate was found to be inhibitory, although the mode of inhibition was not determined.

RESULTS

RNA polymerase activity could not be detected in crude preparations of L. mexicana. It appears that the removal of DNA and inhibitory proteins is necessary to detect this enzyme. In our experiments 96%-99% of the protein concentration was removed from the initial preparation as well as the DNA before RNA polymerase could be detected. The method utilizing poly (ethylene imine) precipitation provides a rapid procedure for removing inhibitory substances. Poly (ethylene imine) is very basic and is expected to precipitate acidic molecules including nucleic acids, nucleoproteins (chromatin and ribosomes), and acidic proteins (including RNA polymerase II) (21). The results of precipitating RNA polymerase with a 10% poly (ethylene imine) solution are shown in Fig. 8. The enzyme activity in the supernatant initially increases, reaches a maximum and then decreases. The initial increase results from the removal of one or more substances which inhibit RNA polymerase. The subsequent decrease in activity reflects the coprecipitation of the enzyme with the poly (ethylene imine), as has been shown to be the case with other systems (21). Fig. 8 shows that all the RNA polymerase activity is effectively precipitated at any concentration of poly (ethylene imine) above 0.4% (v/v) crude extract. Addition of 30 μ l of a 10% poly (ethylene imine) solution ml^{-1} of crude extract removed 97%-99% of the protein and nucleic acids.

The supernatant fluid from the poly (ethylene imine) precipitation was subjected to DEAE-Sephadex chromatography and yielded only one active RNA polymerase peak. Also, the method of Smith and Braun (22) utilizing poly (ethylene imine) precipitation of RNA polymerase activity and subsequent release of RNA polymerase activity with $(\text{NH}_4)_2\text{SO}_4$ failed to detect more than one RNA polymerase when chromatographed on DEAE-Sephadex. Chromatography on DEAE-Sephadex appears to be the most effective means for resolution of the various enzymes in a single chromatographic step. RNA polymerases I, II and III from most cell types are eluted from this ion-exchange matrix at concentrations of approximately 0.05-0.15, 0.15-0.25 and 0.20-0.35 M ammonium sulfate respectively (23). Since we wanted a method which would allow us to detect all three polymerases poly (ethylene imine) precipitation was abandoned. It appeared that this method was inadequate for the solubilization of all RNA polymerase types in *L. mexicana* promastigotes.

To determine the effect of sonication on releasing RNA polymerase activity, we sonicated the crude homogenate for different lengths of time, precipitated inhibitory material with 30 μl poly (ethylene imine) ml^{-1} of crude homogenate, centrifuged ($7,500 \times g$ for 5 min), and assayed for RNA polymerase activity. Fig. 9 demonstrates that sonication is necessary to release RNA polymerase activity, but once the enzyme(s) are released, they are inactivated by further sonication.

We found chromatography on Heparin-Sepharose after homogenation, sonication and protamine sulfate treatment of the crude extract to be a very efficient step in the purification of RNA polymerase. The protein concentration was reduced by 95%. RNA polymerase was eluted with a 0.5 M $(\text{NH}_4)_2\text{SO}_4$ step gradient in Buffer A. The inhibitory substances were removed and one peak of RNA polymerase activity was detected (Fig. 10). Pooling of active fractions from the Heparin-Sepharose and subsequent chromatography on DEAE-Sephadex increased the specific activity 66 fold (Fig. 11). Further chromatography on Carboxymethyl-Sephadex (CM) of pooled active fractions resulted in a 2.25 fold increase in specific activity from the previous step (Fig. 11). Table 3 shows a purification scheme for RNA polymerase. During Heparin-Sepharose and CM-Sephadex chromatography only one RNA polymerase peak was observed. Several times during DEAE-Sephadex chromatography a small RNA polymerase peak could be detected closely associated with the major RNA polymerase peak. Assay of each of the peak fractions, revealed similar sensitivities to α -amanitin and $(\text{NH}_4)_2\text{SO}_4$ concentrations. It is not known whether the appearance of this second peak is the result of proteolysis of the enzyme or represents a multiple form of an α -amanitin-sensitive RNA polymerase.

The results of gel electrophoresis under nondenaturing conditions of RNA polymerase which had been subjected to protamine sulfate treatment, Heparin-Sepharose, DEAE-Sephadex, and phosphocellulose chromatography is shown in Fig. 13. Only two protein bands could be detected when stained with commassie blue. RNA polymerase activity could not be detected when gels were sliced and assayed immediately after electrophoresis. Gel filtration on Sephacryl S-300 of RNA polymerase pooled from a CM-Sephadex column revealed a molecular weight of 360,000 (Fig. 14,15). Since this column did not contain glycerol which stabilizes the polymerase the specific activity after gel filtration was very low. Gel filtration with 25% glycerol in Buffer A takes almost 48 hr to complete. Because of this, the glycerol was omitted, but added to the fractions after filtration.

Fig. 8

Polymin P Precipitation of RNA Polymerase

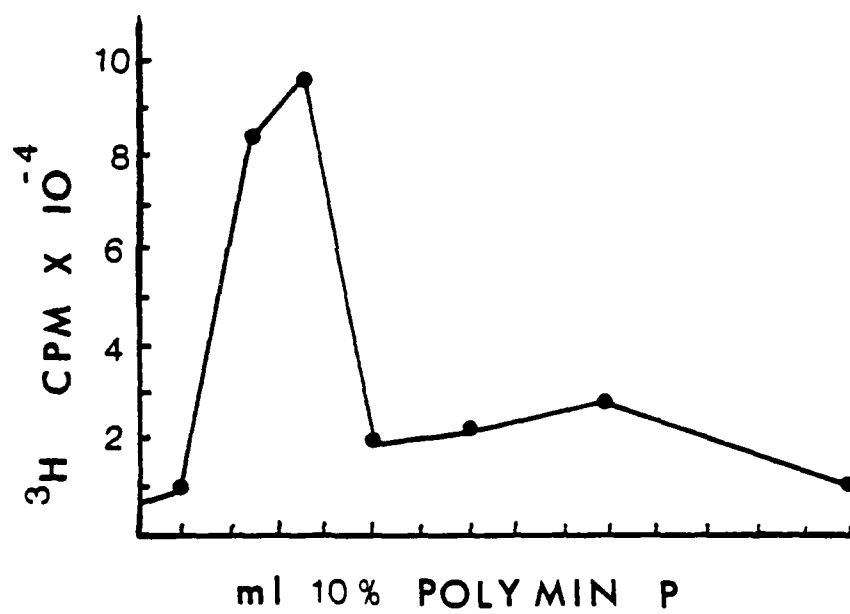


Fig. 9

The Effect of Sonication on RNA Polymerase Activity

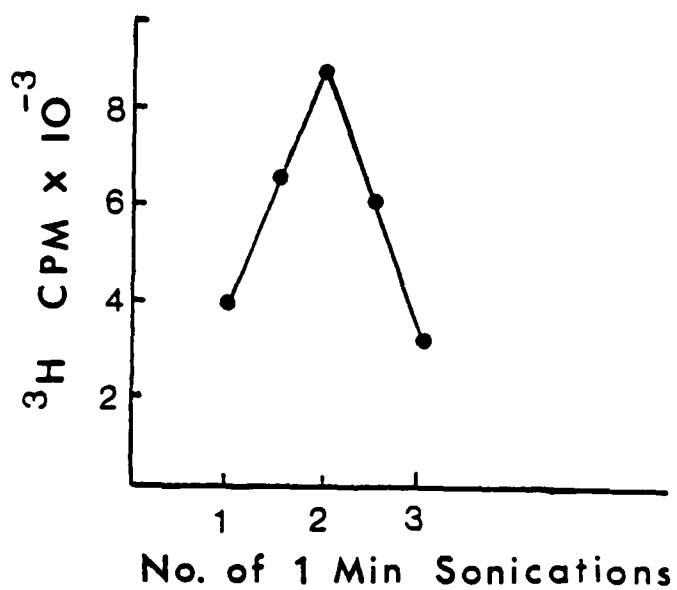
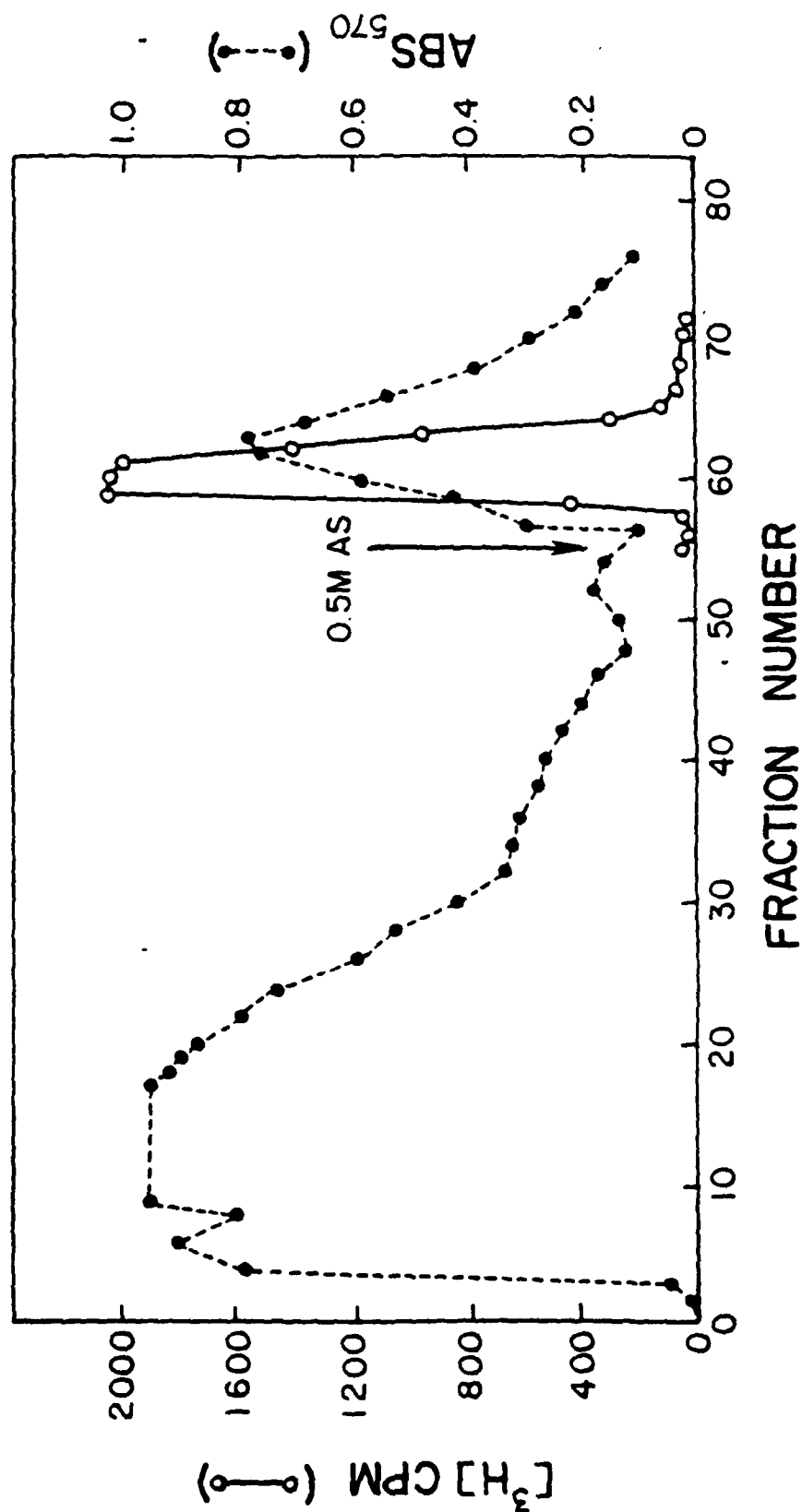
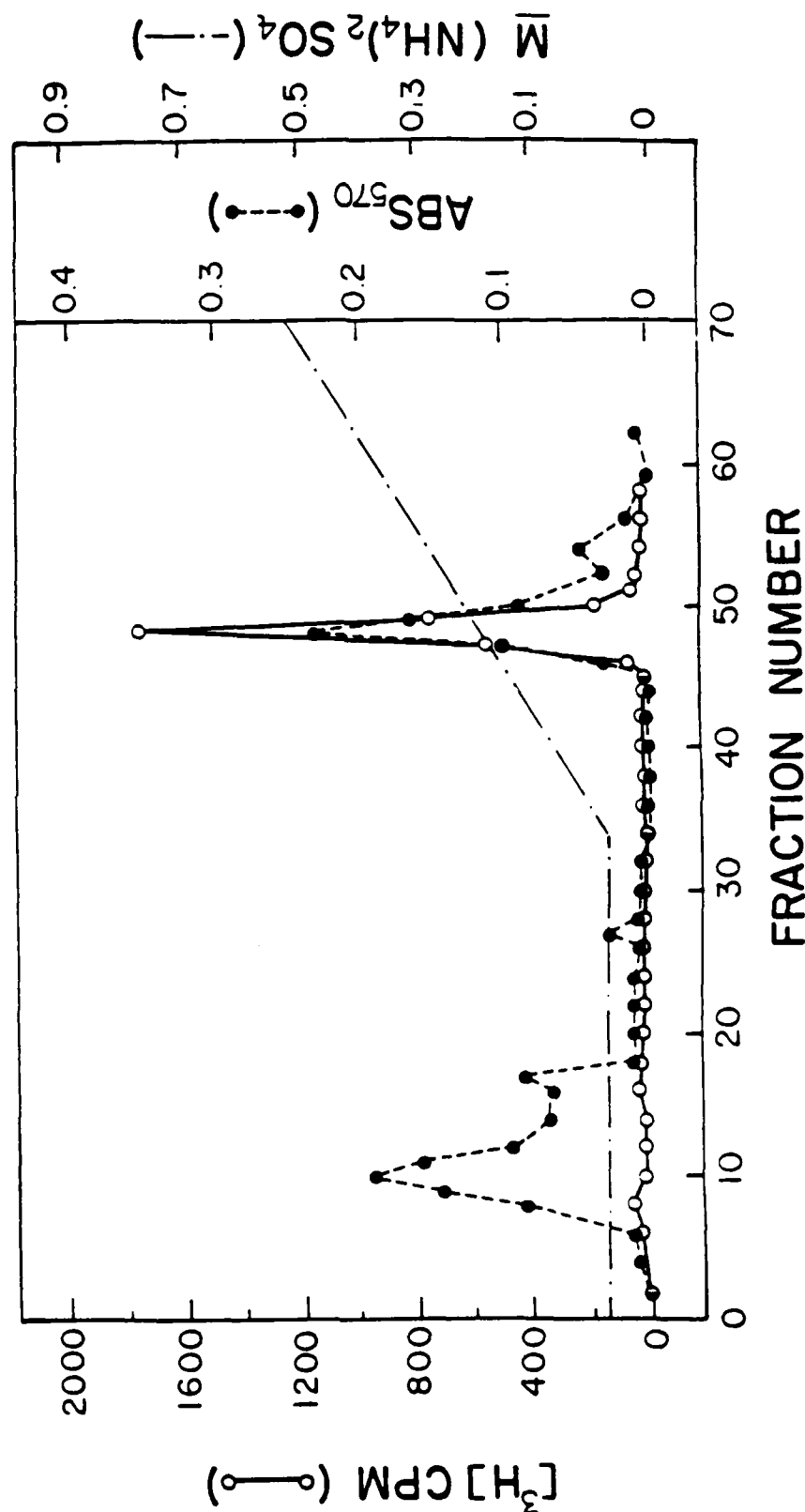


Fig. 10



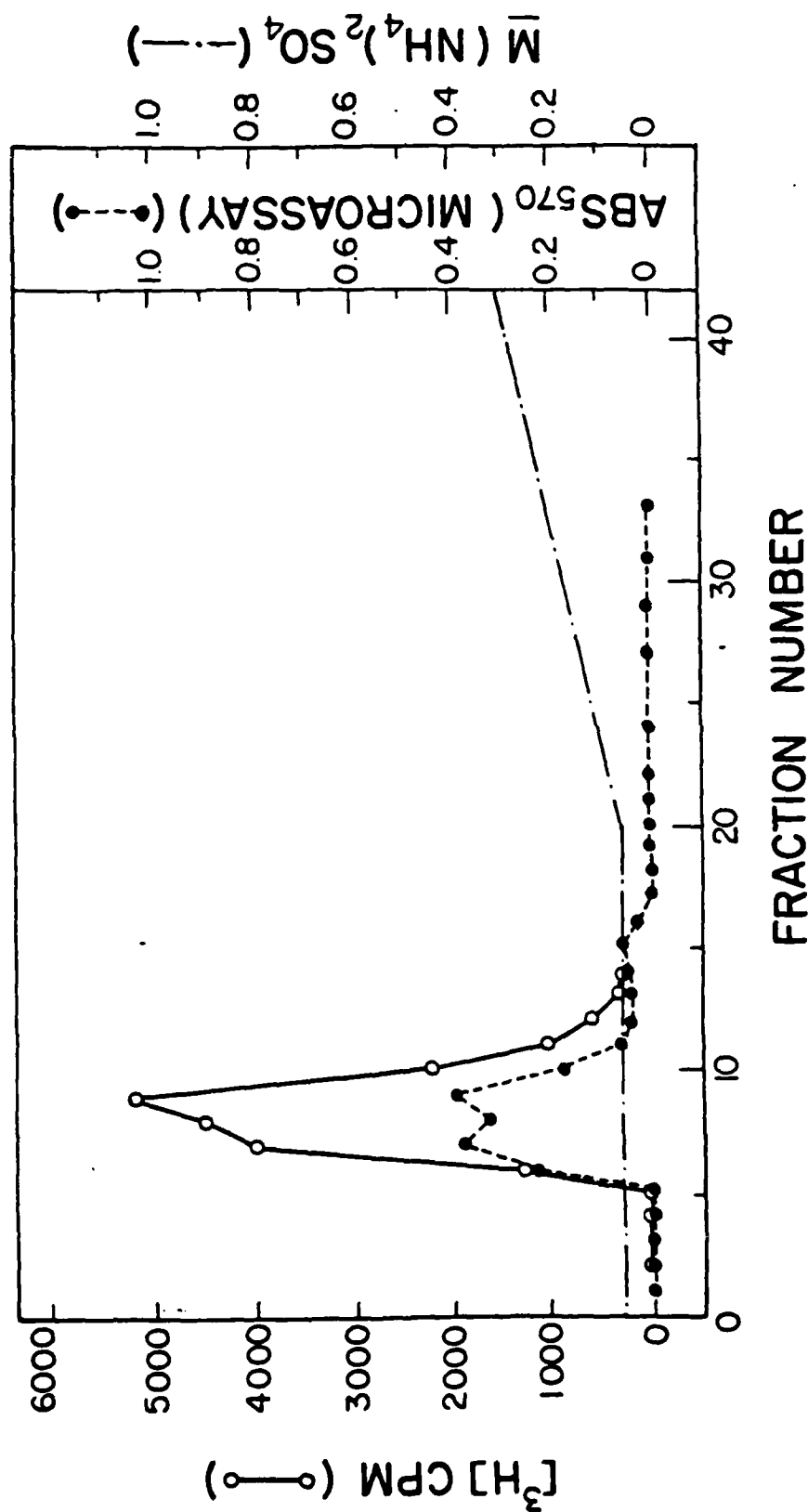
Heparin-Sepharose chromatography of crude extract. Protein 453 mg was applied to a 1.5 x 7.0 cm column of Heparin Sepharose and RNA polymerase was eluted with a step gradient 0.5 M $(\text{NH}_4)_2\text{SO}_4$ in Buffer A.

Fig. 11



DEAE-Sephadex chromatography of pooled active fractions (23 mg protein) from Heparin-Sephadex chromatography. RNA polymerase eluted at 0.25 M $(\text{NH}_4)_2\text{SO}_4$ in Buffer A.

Fig. 12



Carboxymethyl-Sephadex chromatography of active fractions (2.4 mg protein) from DEAE-Cellulose chromatography. RNA polymerase eluted in the void volume at 0.05 M $(\text{NH}_4)_2\text{SO}_4$ in Buffer A.

Table 3 Purification scheme for RNA-polymerase III

Purification Step	Total Volume (ml)	Total Protein (mg)	Units* (ml) ⁻¹	Total Activity (units)	Specific Activity ₂₁ (units mg ⁻¹)
Whole sonicate	19.3	768	Non detectable	-	-
Crude extract (15,000 rpm, 30 min)	15.0	498	Non detectable	-	-
Protoamine sulfate treatment	16.3	453	Non detectable	-	-
Heparin-Sepharose	6.7	23	2.1	14.1	0.6
DEAE-Sephadex	6.0	2.4	15.8	94.8	39.5
CM Sephadex	8.5	1.7	17.7	150.5	88.5

*One unit of activity is defined as the incorporation of 1 pmole of UMP into RNA in 20 min under standard assay conditions. Assay conditions are described under Methods.

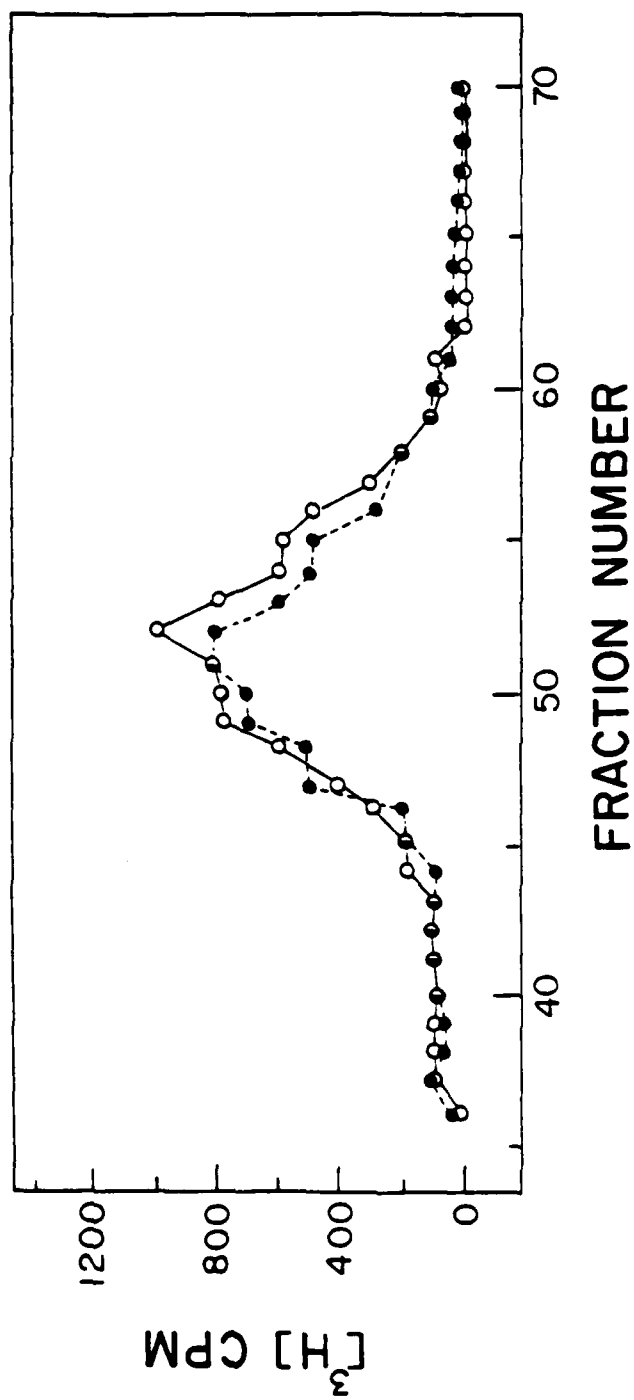
Fig. 13

Polyacrylamide Gel Electrophoresis Under Nondenaturing Conditions of RNA Polymerase



Polyacrylamide gel electrophoresis under nondenaturing conditions of RNA polymerase (104 μ g protein) which was isolated by Heparin-Sepharose, DEAE-Sephadex and phosphocellulose chromatography.

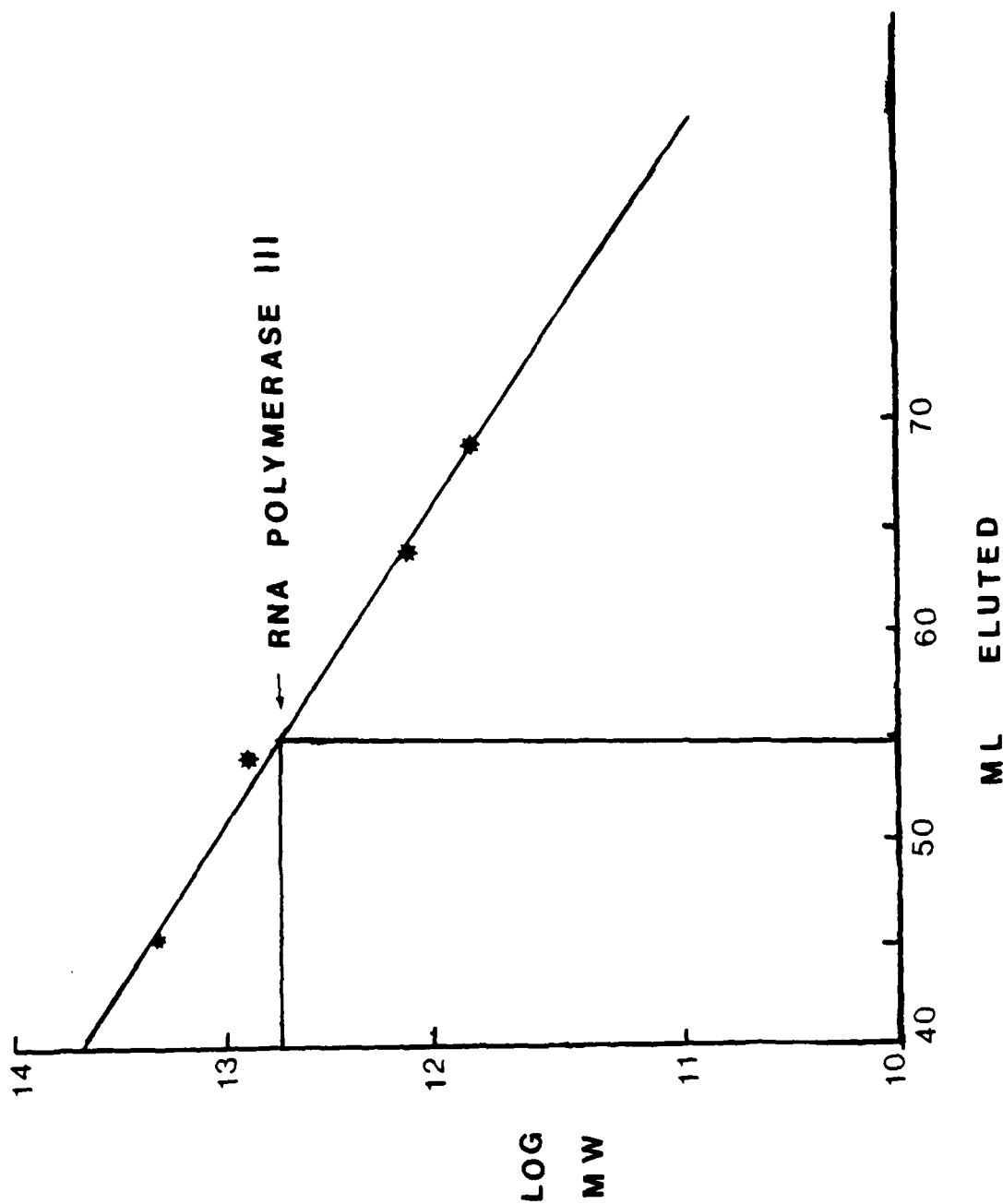
Fig 14



Sephacryl S-300 gel filtration of RNA polymerase isolated from DEAE-Sephadex chromatography. ○—○ No α-Amanitin, ●—● activity in the presence of 1 μg/ml α-Amanitin (RNA polymerase II is sensitive to this concentration).

Fig. 15

ESTIMATION OF MOLECULAR WEIGHT OF RNA POLYMERASE



Values for standard proteins are plotted against their elution volumes. Standard mw of proteins were thyroglobin (669,000), apoferritin (443,000), B-amylase (200,000) and alcohol dehydrogenase (150,000). The elution position of RNA polymerase is indicated. (360,000)

Assay of RNA polymerase from the pooled fractions of a DEAE-Sephadex column revealed that the enzyme reaction was linear up to 20 min (Fig. 16). RNA polymerase isolated from DEAE-Sephadex chromatography showed 50% inhibition at a 4 $\mu\text{g/ml}$ α -amanitin concentration (Fig. 17). The α -amanitin up to 20 $\mu\text{g/ml}$ did not completely inhibit this enzyme. This same enzyme preparation dialyzed against Buffer A without metal ions was tested for optimum metal ion concentration in the RNA polymerase assay. Fig. 18 shows that the optimum concentration for MnCl_2 was 1 mM and that increasing the MnCl_2 concentration beyond 3 mM greatly inhibited the enzyme. The enzyme showed optimal activity with a MgCl_2 concentration between 2-4 mM, but this metal was only 14% as effective in enhancing optimal activity when compared to MnCl_2 .

DEAE-Sephadex pooled fractions dialyzed against Buffer A without $(\text{NH}_4)_2\text{SO}_4$ were used to determine the optimum salt concentration for the RNA polymerase assay. As shown in Fig. 19, an $(\text{NH}_4)_2\text{SO}_4$ concentration between 0.02-0.06 M gave optimal RNA polymerase activity. Increasing concentrations beyond 0.1 M $(\text{NH}_4)_2\text{SO}_4$ inhibited the enzyme.

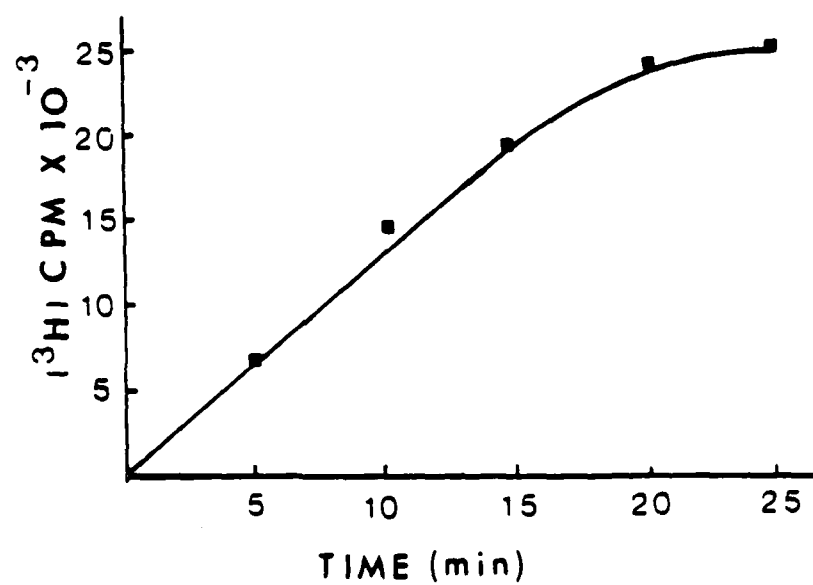
Since the purpose of our isolation of RNA polymerase was to provide an adequate system to test promising nucleotide analogs, we tested the effect of formycin A-triphosphate (FoA-TP) and cordycepin triphosphate on RNA polymerase isolated from DEAE-Sephadex chromatography. As shown in Fig. 20, FoA-TP appears to be a competitive substrate for the RNA polymerase. The addition of increasing concentrations of ATP (to 0.6 mM) in the presence of 0.4 mM FoA-TP relieves the competition, but does not completely reverse it. We have demonstrated previously that FoA-TP is incorporated into RNA and that the mRNA containing FoA nucleotides translates less efficiently than normal mRNA in *L. mexicana* promastigotes (24). We are in the process of determining the effect of tRNA containing FoA nucleotides on tRNA function.

Cordycepin triphosphate is known to affect RNA polymerase in a variety of organisms, but the exact kinetic nature of this inhibition is unknown (25). Cordycepin triphosphate inhibited the RNA polymerase in a linear manner from 10-60 μM , but increasing the concentration up to 100 μM did not completely inhibit this enzyme. This result may be due to more than one RNA polymerase species present in our preparation showing different sensitivities to this nucleotide, or the nucleotide may compete with the natural nucleotides (which are all present in the assay) and under these conditions was not able to bind to all the available sites.

The enzyme isolated has characteristics of true DNA-dependent RNA polymerase in that it requires DNA and all four nucleoside triphosphates for the synthesis of RNAase-sensitive products. The reported ammonium sulfate optima and metal ion optima, as well as relative activities of the enzyme with Mn^{2+} versus Mg^{2+} are similar to those reported for RNA polymerase III (26). Also, α -amanitin sensitivity and elution characteristics during chromatography indicate that this enzyme is RNA polymerase III (27).

RNA polymerase III is present in a variety of cells and tissues although its concentration is subject to great variability (26). Eukaryotic cells contain several distinct RNA polymerase proteins, which are localized in different sub-cellular fractions and play different functional roles in the cell. DNA-dependent RNA polymerase III represents one of the three major classes of nuclear RNA polymerases in eukaryotes. The class III RNA polymerases from a variety of cell types have been shown to have characteristic catalytic and chromatographic

Fig. 16



RNA polymerase activity in response to time. Assay conditions are described in Methods. Activity was dependent on DNA, all four nucleotide triphosphates, and the enzyme products were degraded by RNAase.

Fig. 17

Effect of α -amanitin upon the activity of RNA polymerase eluted from DEAE-Sephadex chromatography.

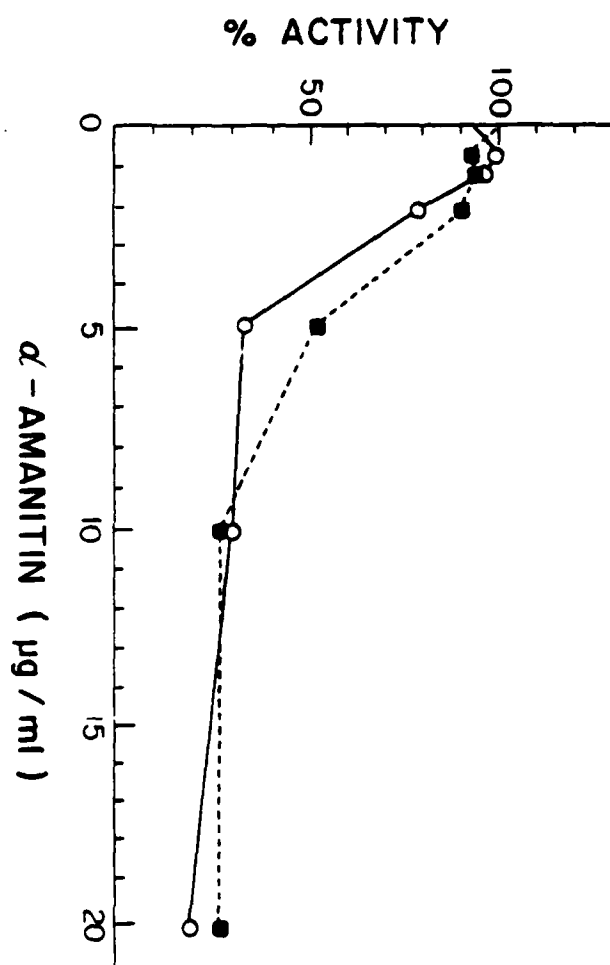


Fig. 18

Effects of Mn^{2+} ● and Mg^{2+} □ upon the activity of RNA polymerase eluted from DEAE-Sephadex chromatography.

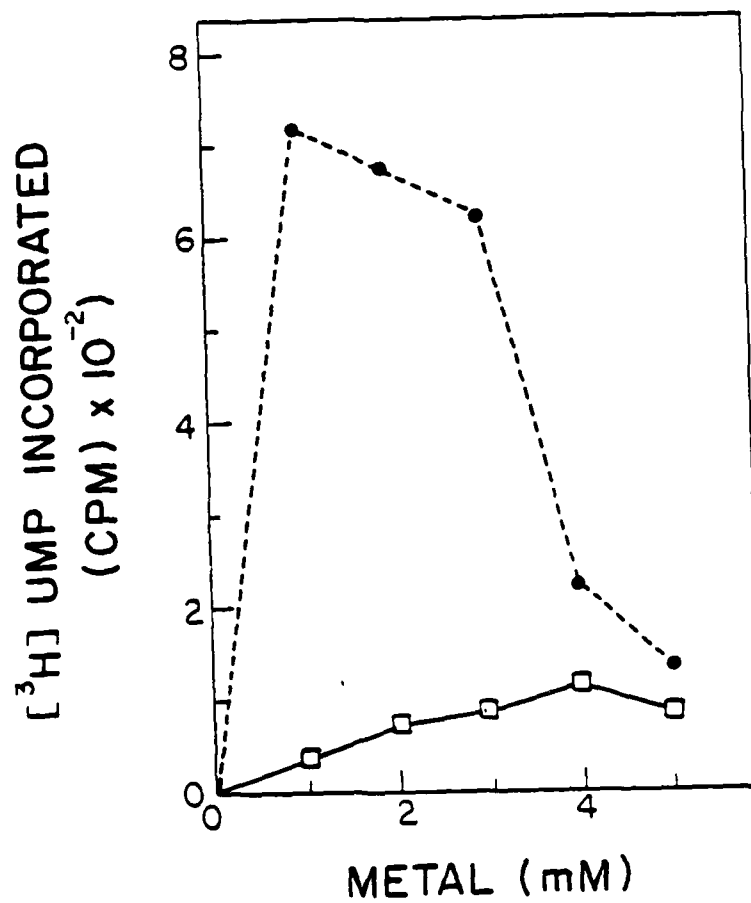


Fig. 19

Effect of ammonium sulfate upon the activity of RNA polymerase eluted from DEAE-Sephadex chromatography.

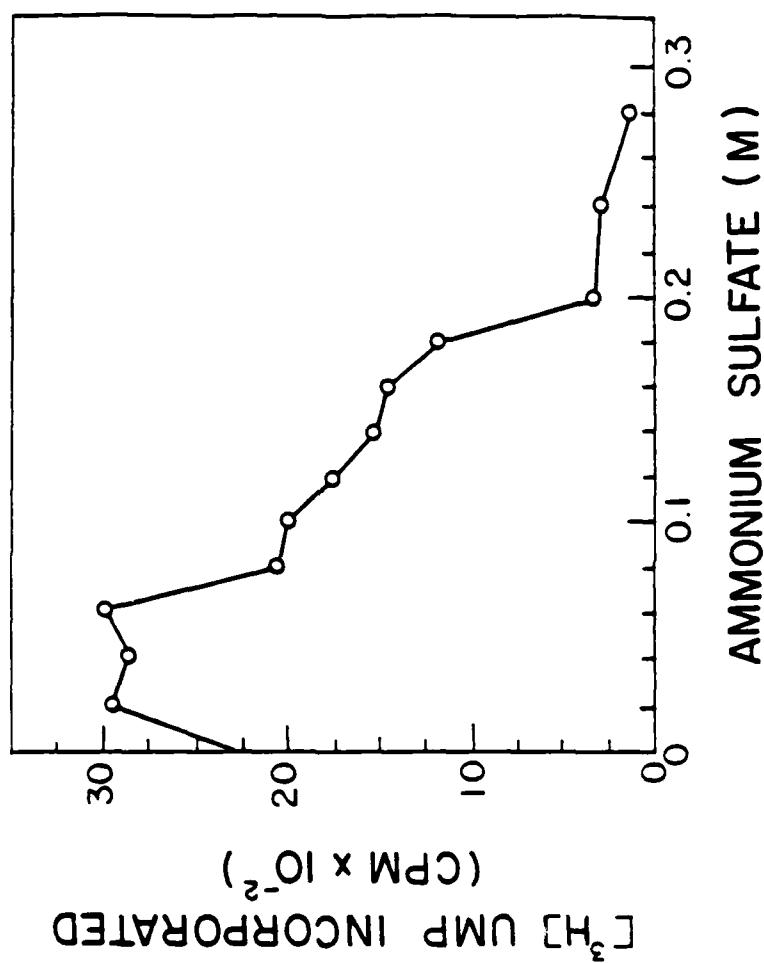
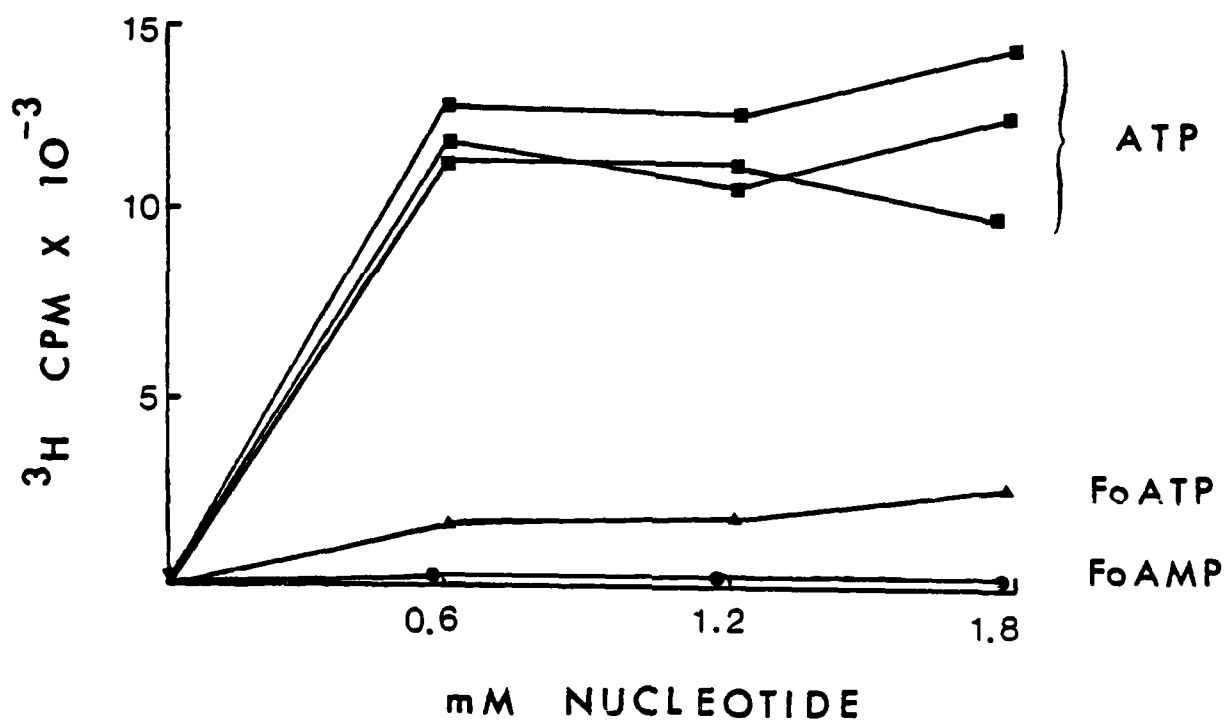


Fig 20

The Ability of Different Nucleotides to Serve as a Substrate for
RNA Polymerase



properties and characteristic subunit structures, all of which distinguish these enzymes from the homologous class I and II RNA polymerases (26). The class III RNA polymerases have been implicated in the synthesis of tRNA, 5 sRNA, and other small cellular RNAs and in the synthesis of two small viral RNA species in adenovirus-infected cells (26).

We have tested the following compounds sent by WRAIR on isolated RNA and DNA polymerase. Our lack of inhibition probably results from the fact that many of these compounds are active when phosphorylated to the triphosphate.

Compounds Tested with No Effect on DNA Polymerase (500 μ m)

BK86124 Allopurinol riboside
BK74731 Oxoformycin
BK63005 3-B-D-Ribofurano sylpyrazolo-[4,3-d] pyrimidine-7-thione
BK48464 6-Aminoallopurinol riboside
BK71338 Oxoformycin A

Compounds Inhibitory to DNA Polymerase at 500 μ m

BK63863 11.52% Thiopurinol riboside

Compounds Inhibitory to RNA Polymerase at 500 μ m

BK74731 9.5% Oxoformycin B
BK71338 13.7% Oxoformycin A

INCORPORATION OF FORMYCIN B AND ITS METABOLITES
INTO THE RNAs OF LEISHMANIA MEXICANA

In experiments to elucidate the mechanism of action of formycin B (FoB), we isolated total RNA from [³H]-FoB exposed cells (8 μ M for 6 hr) and fractionated this into tRNA, rRNA and mRNA by a sucrose gradient (15%-30%). Figure 21 demonstrates that [³H]-FoB metabolites were incorporated into all RNA species.

In a previous publication, Biochemistry International 9(2), 207-218, we demonstrated for the first time that mRNA of leishmanial cells exposed to Formycin β was defective in its ability to function in the translational process. We now have isolated mRNA, rRNA and tRNA from [³H]-Formycin B exposed cells and have demonstrated for the first time that it is converted to Formycin A and preferentially incorporated into the mRNA as opposed to the other RNAs. This data substantiates the hypothesis that one of the targets of Formycin B toxicity in leishmania is via defective mRNA translational capabilities. Substantial amounts of Formycin B as well as Formycin A were found in the mRNA and rRNA (Figs. 22,23). Our washing and isolation procedures were quite extensive, and the possibility exists that Formycin B nucleotides are incorporated into these RNAs. If the incorporation of Formycin B nucleotides takes place and alters RNA function, this would help explain Dr. Buddy Ullman's mutant data suggesting that Formycin B compounds are toxic (28).

Fig. 21

SUCROSE GRADIENT SEPARATING RNAs FROM TOTAL RNA

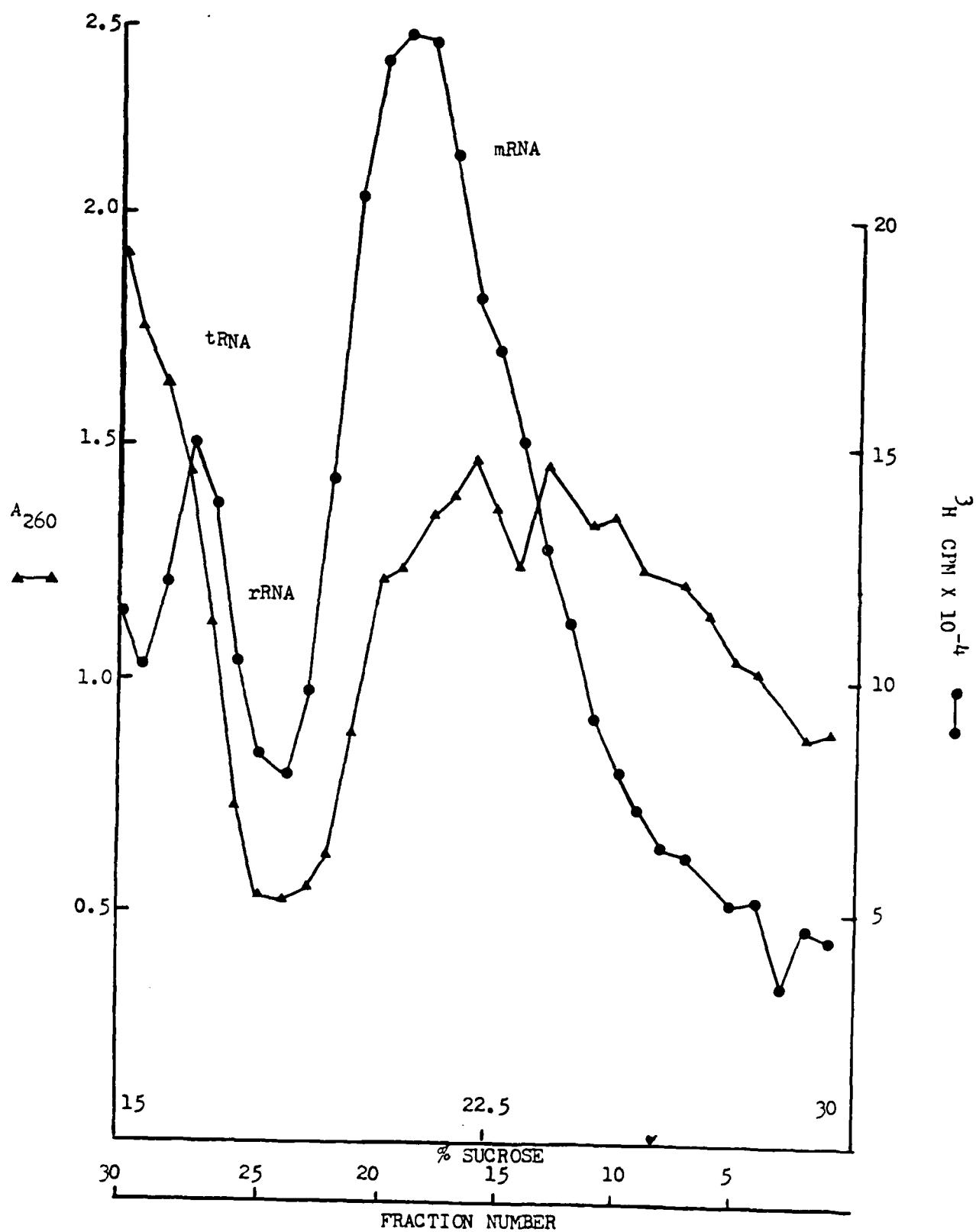


Fig. 22

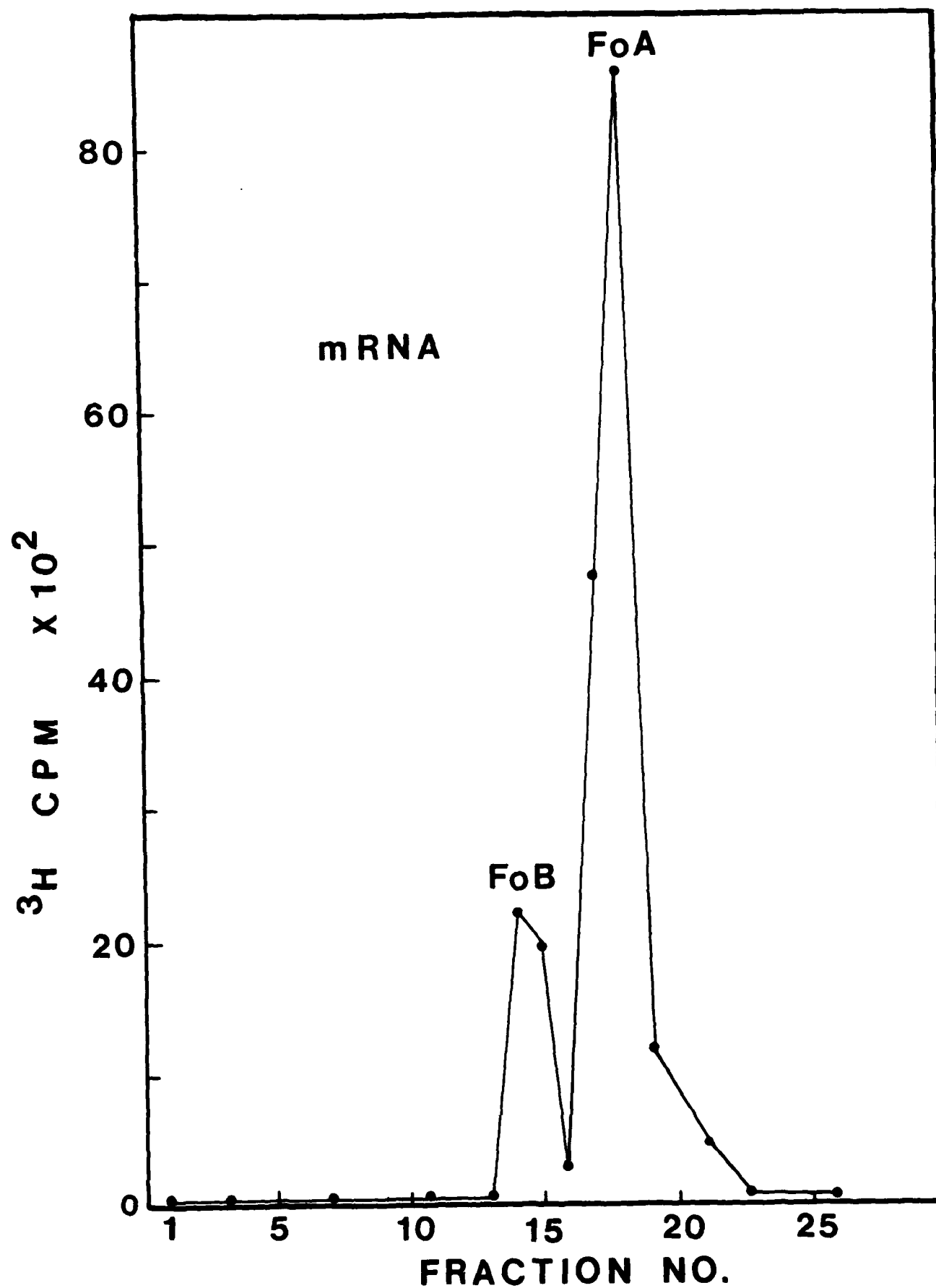
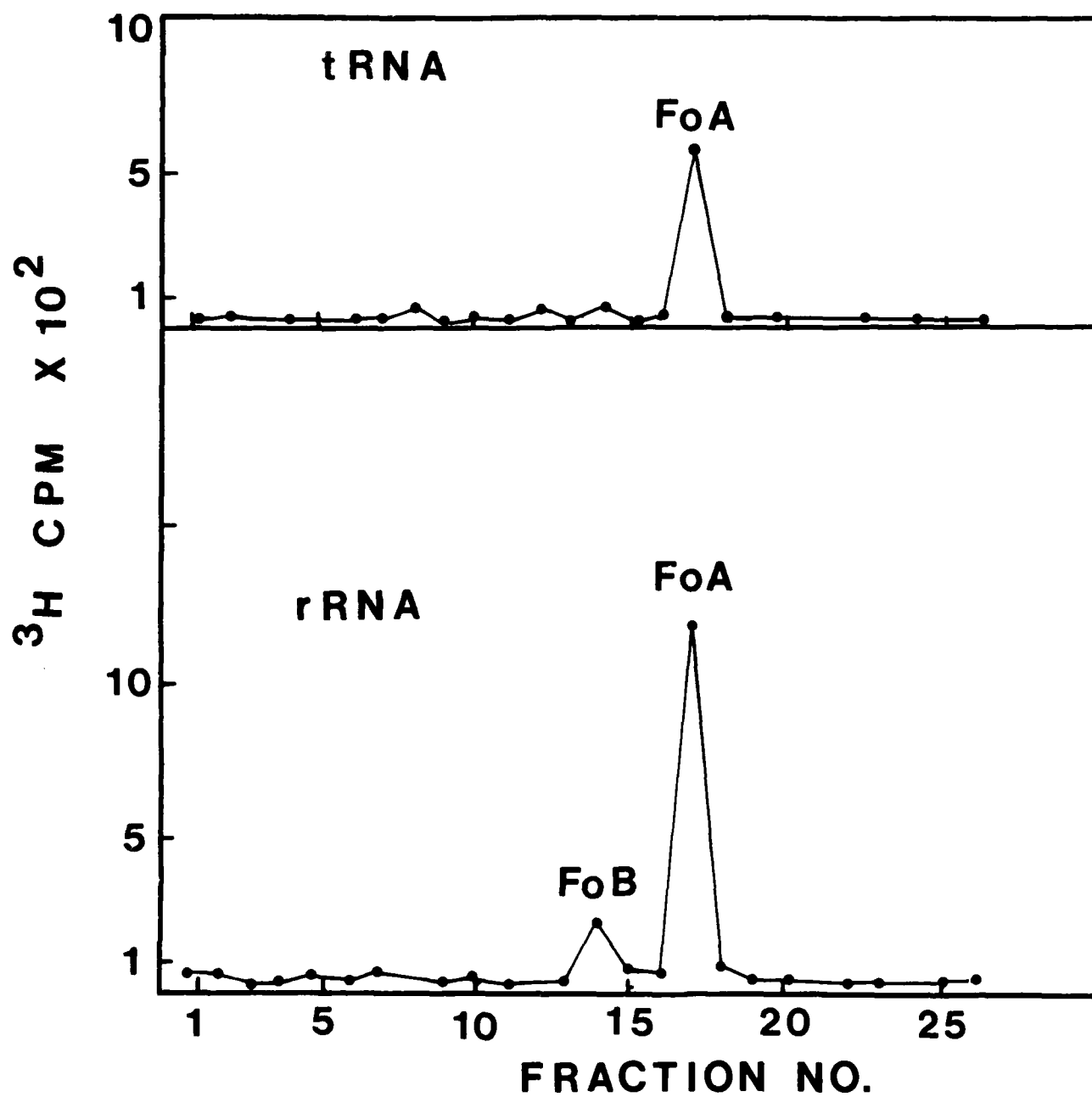


Fig. 23



In brief, L. mexicana promastigotes were exposed to [H^3] Formycin B 8 μ m for 6 hrs., harvested, washed and lysed. mRNA, tRNA and rRNA were isolated as described in Methods in Enzymology, vol. XII, pp. 581-596. The RNAs were digested to the nucleosides by the method of Randerath and Gupta (29) and Gupta and Randerath (30) and analyzed by HPLC by the method of Hartwick et al. (30). Figs. 22 and 23 show the results of this analysis. The major peak eluted corresponds to Formycin A (FoA) and the minor peak Formycin B (FoB). Based on 3H counts/ μ g RNA, 93% of the counts represented FoA in mRNA, 3.6% in tRNA, and 3.3% in rRNA. Of the total counts found in mRNA, 16% were represented by FoB and 84% by FoA. In tRNA, 10% of the counts eluted with FoB, and 90% with FoA. For rRNA, 19% of total counts in rRNA eluted with FoB, and 81% with FoA.

INHIBITION OF LEISHMANIAL DNA SYNTHESIS BY SINEFUNGIN

SUMMARY

RNA, DNA and protein biosynthesis were studied in Leishmania donovani and L. tropica promastigotes cultured with or without sinefungin. Only thymidine incorporation was significantly impaired by this compound. Neither the uptake of thymidine nor its phosphorylation was inhibited. Analysis of the DNA indicates that the inhibition of thymidine incorporation affects mostly nuclear DNA, while kDNA is weakly affected by this drug. No such effect on thymidine incorporation was observed in macrophages, the host cells of these parasites.

INTRODUCTION

Sinefungin 1 (Fig. 24), a natural nucleoside isolated from cultures of Streptomyces incarnatus and S. griseolus is an antifungal and antiparasitic agent in in vitro and in vivo (31-40).

This nucleoside is structurally related to S-adenosylmethionine (SAM) and to S-adenosylhomocysteine (SAH), respectively substrate and inhibitor of methyltransferases, and was shown to inhibit these enzymes in various cells (41-44). However contrary to what was expected, sinefungin is a poor inhibitor of leishmanial protein methylases in vitro and in vivo (40 and Paolantonacci et al. in preparation). As the growth inhibition provoked by sinefungin may be related to other cellular events we studied the effect of this nucleoside on macromolecular synthesis in promastigotes of Leishmania. Our results showed that only NA synthesis was drastically inhibited in cells grown in the presence of sinefungin and that this inhibition was not the consequence of either nucleoside uptake or its phosphorylation.

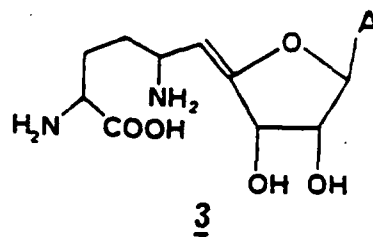
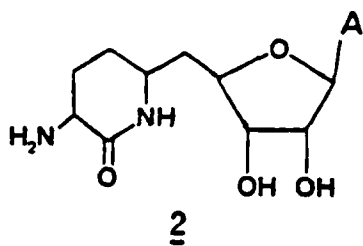
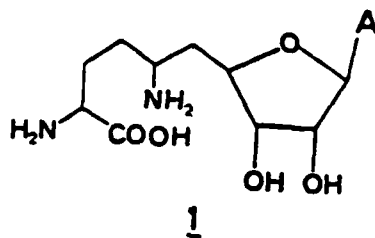
MATERIAL AND METHODS

Growth of organisms: Leishmania tropica (strain LRC-32) and L. donovani (strain LRC L-52) originated from the strain collection of the World Health Organization's International Reference Center for leishmaniasis (WHO-LRC). The promastigotes were grown in a semi-defined medium as described previously (40).

Effect of sinefungin on macromolecular synthesis

Promastigotes were cultured with and without sinefungin (40) for various lengths of time, and then the appropriate radioactive precursor was added for one hour to the medium: L-[4,5-³H]leucine, 138 Ci/mM, (5μCi/ml) or [5-³H]-uridine, 30 Ci/mM, (2.5μCi/ml) or [methyl-³H]thymidine, 42 Ci/mM, (2.5μCi/ml) (Amersham G.B.). After one hour labelling, the cells were centrifuged and rinsed twice in cold phosphate buffered saline (PBS). To measure the uptake into the soluble pool and the incorporation into nucleic acids and proteins, the

Fig. 24



- 1 = sinefungin
2 = cyclic sinefungin
3 = A9145C

method of Schmidt and Tannhauser (45) was used. Protein concentration was determined by the Lowry procedure (46).

The phosphorylated intermediates of nucleosides were identified according to the method of Cass and Paterson (47). DNA was extracted as described by Blin and Stafford (48).

DNA analysis of equilibrium sedimentation in CsCl gradients was carried out as described by Simpson and Berliner (49).

Sinefungin and A9145-³C were kindly provided by Dr. R. Nagarajan (Lilly Research Laboratories, Indianapolis, USA). The cyclic derivative of Sinefungin² (Fig. 26) was synthesized in our laboratory by Dr. M. Geze (50).

RESULTS

Effects of sinefungin on macromolecular biosynthesis:

RNA, DNA and protein biosynthesis were studied in the absence and in the presence of sinefungin concentrations which inhibit promastigote multiplication (0.26 μ M and 0.26 μ M) (31). As shown in Table 4 only thymidine incorporation was significantly impaired in *L. donovani* promastigotes. After 6 h in the presence of 0.026 μ M and 0.26 μ M sinefungin the incorporation of thymidine was inhibited by 70 and 91 percent respectively. Under these conditions thymidine uptake into the cells was not affected. Uridine incorporation and uptake were both moderately decreased; however the uptake of uridine into the TCA-soluble fraction was inhibited to a lesser degree than the incorporation into TCA-insoluble material. Thus, a slight effect on RNA synthesis cannot be ruled out. Leucine incorporation into protein was unaffected by the drug under these conditions.

Characteristics of the inhibition of DNA synthesis by sinefungin and analogues:

As shown in Table 5 the inhibition of thymidine incorporation was time and concentration dependent in both *Leishmania* species tested. The uptake of the precursor was unaffected. The cyclic lactame inhibited the DNA synthesis in both species but at a much higher concentration. 0.26 μ M A 9145 C strongly inhibited DNA synthesis in *L. tropica* but had no effect on *L. donovani*. SAH was ineffective, probably due to its rapid metabolism.

The inhibition by low doses of sinefungin on DNA synthesis seemed specific for the parasites since in macrophages (host cells) no such effect on thymidine incorporation could be observed with 10 to 100 times higher concentrations with incubation times up to 24 h (results not shown).

T A B L E 4

Macromolecular biosynthesis in L.donovani promastigotes cultured with or without sinefungin.

		CPM		
Labelled precursor	T C A *	Sinefungin concentration (μ M)		
	fraction	0	0.026	0.26
Leucine	soluble	24 640 (100)	32 390 (131)	28 750 (117)
	insoluble	59 680 (100)	77 250 (129)	61 980 (104)
Uridine	soluble	1 234 280(100)	945 050 (77)	633 260 (51)
	insoluble	492 300(100)	314 630 (64)	192 590 (39)
Thymidine	soluble	963 060 (100)	984 000(102)	1 207 270(125)
	insoluble	511 830 (100)	151 970 (30)	44 280 (9)

Leishmania donovani promastigotes were cultured with or without sinefungin; after 6 hours the cultures were labelled for 1 hour with radioactive precursors : 5 μ Ci/ml for leucine and 2,5 μ Ci/ml for uridine or thymidine. Then cells were centrifuged, rinsed in Phosphate Buffered Saline (PBS) and treated with 5% cold TCA* as described in (45) . Results are expressed as cpm/mg proteins. Values in brackets represent the percentage of incorporation with respect to the control.

*Trichloroacetic acid

TABLE 5

Effect of sinefungin and some analogues on thymidine uptake and incorporation in L.donovani and L.tropica promastigotes.

Compound	Conditions		<u>L.donovani</u>		<u>L.tropica</u>	
	μ M	time*	TCA-soluble	TCA-insoluble	TCA-soluble	TCA-insoluble
Sinefungin	0.26	1	118	50	164	84
	"	2	148	31	108	46
	"	4	179	15	132	30
	"	6	126	9	135	17
	0.026	1	101	73	164	112
	"	2	95	61	108	78
	"	4	168	49	85	32
	"	6	100	37	72	25
DL cyclic-sinefungin	5.20	6	139	16	113	11
	0.52	6	101	60	76	41
A 9145C	2.60	6	138	17	99	8
	0.26	6	107	101	106	37
SAH	2.60	6	69	107	-	-

* time of exposure before labelling (in hours)

Cultures were treated with two concentrations of sinefungin or analogues for various length of time, and then labelled with 2,5 μ Ci/ml of thymidine for 1 h. Cells were then washed and processed as described under material and methods. Results are expressed as the percentage of specific radioactivity (cpm/mg of proteins) with respect to untreated cells.

Analysis of DNA synthesized in control and treated cells:

Analysis of DNA synthesized in the presence or absence of sinefungin on CsCl gradient is shown in Fig. 25. The amount of thymidine incorporated in nuclear DNA (n DNA) is inhibited by 80% and that of kinetoplast DNA (k DNA) by 50% in cells treated with 0.26 μ M sinefungin for 6 hours. The buoyant densities of DNA were estimated at 1.706 and 1.680 for nDNA and kDNA, respectively.

Phosphorylation of nucleosides in cells cultured with or without sinefungin:

In order to know if the inhibition of DNA synthesis is the result of an inhibition of thymidine phosphorylation by the drug we analyzed the phosphorylated nucleoside contents in cells cultured with or without sinefungin. Results are summarized in Table 6. In both species studied there was no inhibition of the nucleoside phosphorylation. Furthermore, a slight accumulation of nucleoside triphosphate was observed in treated cells, except for uridine in L. tropica.

DISCUSSION

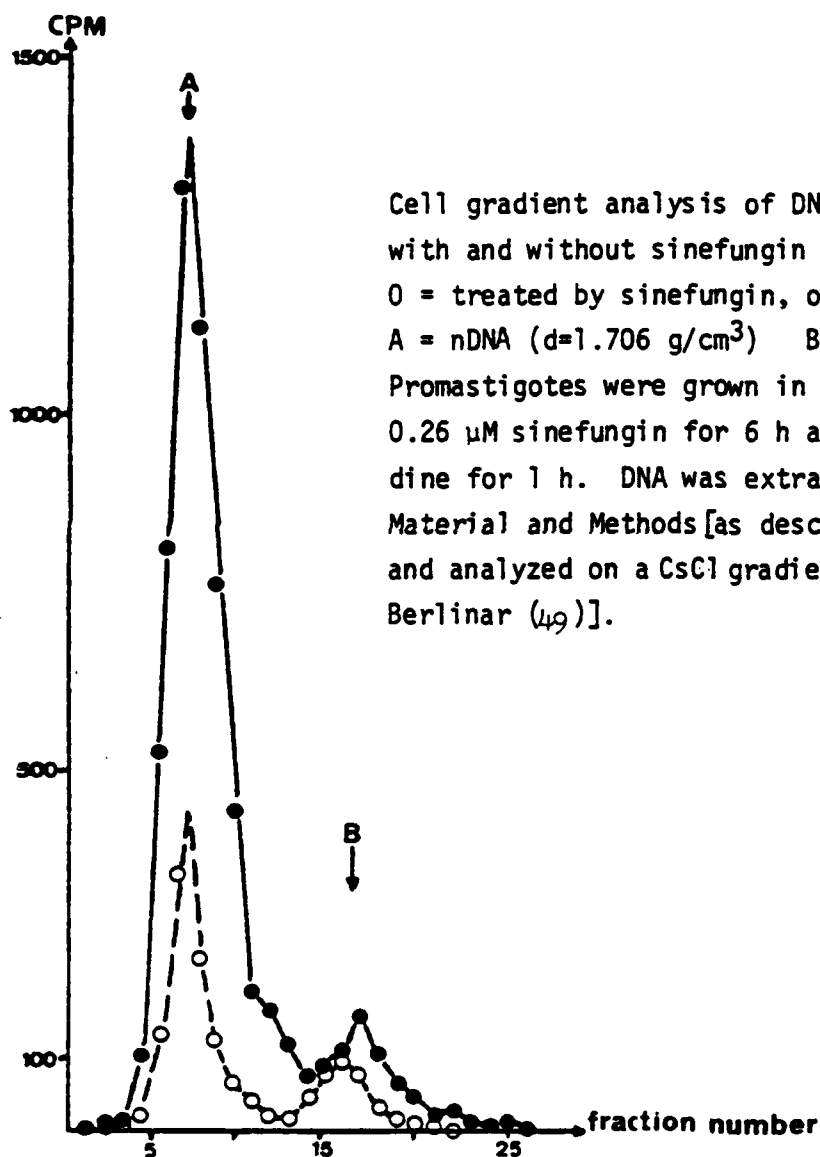
Sinefungin shows antileishmanial activity towards promastigotes (33,38). Our data indicate that this molecule inhibits drastically the incorporation of thymidine into promastigotes DNA but this incorporation is unaffected in macrophages.

The lack of uptake of sinefungin into host cells does not account for the species specificity, since sinefungin is able to penetrate infected macrophages and inhibit amastigote multiplication (33,38). Similarly, the synthesis of DNA in Rous Sarcoma Virus-infected chick embryo fibroblasts was not affected by sinefungin treatment while the transformation of these cells was greatly reduced by this drug (42).

Buoyant density analysis of the DNA of L. donovani has shown that incorporation of thymidine was less inhibited in kDNA than in nDNA. This could reflect either a lower penetration of sinefungin into the kinetoplast or a different sensitivity of the target. The Buoyant density for kDNA and nDNA of L. donovani was similar in control and treated cells. Attempts to explain the mechanism of action of sinefungin have shown that this molecule does not inhibit the phosphorylation of thymidine or other nucleosides in vivo, nor the DNA polymerase activity in vitro (unpublished data).

It has been reported that sinefungin caused actively dividing cells of Saccharomyces cerevisiae to arrest within one cell cycle as unbudded cells. These cells were blocked in the cell initiation step "start" (54). The active concentration range was of the same order of magnitude in both cases: 0.7 μ M for yeast (51) and 0.26 μ M for Leishmania (40). As in yeast, there is a modification of the Leishmania morphology upon treatment: the promastigotes become rounded. Sinefungin-treated cells both in yeast and parasite show: (a) the reversibility of growth inhibition when cells, treated with low concentrations

Fig. 25



Cell gradient analysis of DNA from L. donovani promastigotes with and without sinefungin

0 = treated by sinefungin, o = untreated

A = nDNA ($d=1.706 \text{ g/cm}^3$) B = kDNA ($d=1.680 \text{ g/cm}^3$)

Promastigotes were grown in the presence or in the absence of $0.26 \mu\text{M}$ sinefungin for 6 h and then labelled with $[^3\text{H}]$ -thymidine for 1 h. DNA was extracted and analyzed as described in Material and Methods [as described by Blin and Stafford (48) and analyzed on a CsCl gradient as described by Simpson and Berlinear (49)].

T A B L E 6

Nucleoside phosphorylation in promastigotes cultured with or without sinefungin

Metabolites	<u>L.donovani</u>		<u>L.tropica</u>	
	control	treated	control	treated
TMP	46	27	25	12
TDP	25	16	36	45
TTP	24	47	26	35
UMP	28	20	28	27
UDP	35	34	36	39
UTP	35	35	36	30
AMP	11	6	12	5
ADP	39	28	25	29
ATP	48	62	61	63

Leishmania promastigotes were cultured with or without sinefungin (0.26 μ M) for 6 hours, and then labelled with 5 μ Ci/ml of nucleoside for one hour. Cells were then centrifuged, rinsed with PBS, extracted with perchloric acid 0.5 N, and the extract chromatographed with unlabelled carriers. Spots were localized with UV light, scrapped off and the radioactivity measured.

Results are expressed as percentage of radioactivity found in mono-, di- and tri-nucleosides.

of sinefungin, are transferred into drug-free medium; (b) the effect of the drug is prevented or reversed by S-adenosyl methionine (SAM) added with or after sinefungin (Paolantonacci et al., in preparation). However methionine prevents growth inhibition in yeast but not in Leishmania.

The precise mechanism of inhibition by sinefungin of growth and thymidine incorporation into DNA of Leishmania promastigotes is not yet completely elucidated. The fact that this compound has no effect on protein methylases but can be reversed by SAM suggests that the cellular target of sinefungin in the promastigotes is a specific methylase, probably related to DNA replication. In this respect it is interesting to consider the results of Naguchi et al. (52) describing an enzymatic complex named "replitase." This complex was shown to be nuclear and associated during the S phase in Chinese hamster embryo fibroblasts and dissociated and cytoplasmic during the other phases of the cell cycle. This complex contains DNA polymerase and DNA methylase activities and is fully associated with newly synthesized DNA. Whether such a "replitase"--which can be a potential target for sinefungin--exists in Leishmania is now under investigation in our laboratory.

Culture Methods:

The organisms used in this project have been obtained from the Walter Reed Army Institute of Research through the courtesy of Dr. Joan Decker-Jackson and Dr. Jonathan Berman. The organisms used most have been Leishmania mexicana amazonensis WR 227 and L. donovani WR 130 (Khartoum strain-drug sensitive visceral leishmaniasis). Other organisms presently being cultivated in this laboratory are L. braziliensis WR 424 (Murray isolate from Panama causing cutaneous leishmaniasis), L. braziliensis WR 063 (Terborgh isolate from Peru, causing mucocutaneous leishmaniasis). These organisms are maintained by weekly transfers into Schneider's medium [Grand Island Biological Co., Grand Island, N.Y. (Gibco)] containing 10% heat inactivated fetal bovine serum (HIFBS: GIBCO).

For growing large batches of leishmaniae promastigotes, Brain Heart Infusion Medium (BHI) containing 37 g Difco Brain Heart Infusion/liter water, 10% heat inactivated serum and 26 µg hemin/ml was used. Cells were grown at 26°C in 2000 ml wide Fernback flasks containing 250 ml of BHI and harvested during the exponential growth phase (about day 4).

For defined biochemical experiments the medium of Steiger and Black was used. This medium was used for all transport, uptake and reversal experiments. The cells were depleted of purines by transferring an inoculum from Brain Heart Infusion into Steiger and Black medium with purine omitted, but with 5% heat inactivated fetal bovine serum. The cells were incubated in this medium for 48 hr at 26°C. The cells were then aseptically centrifuged at 5000 x g for 10 min and resuspended to the desired number into fresh Steiger and Black medium Minus purine. By treating the cells in this manner, we avoided as much as possible interference of the metabolism of the compound being tested by the purines in the medium.

RE 1X (Steiger and Black) (53)

Components per liter:

A)	8.0g NaCL	C)	300 mg L-glutamine
	400 mg KCL		1.0 g NaHCO ₃
	200 mg MgSO ₄ .7H ₂ O		14.25 g HEPES (=60 mM)
	60 mg Na ₂ HPO ₄ .2H ₂ O		20 mg adenosine
	60 mg KH ₂ PO ₄	D)	1 mg D-biotin
	2.0g glucose		1 mg choline chloride
B)	200 mg L-arginine		1 mg folic acid
	100 mg L-histidine		2 mg i-inositol
	100 mg L-isoleucine		1 mg niacinamide
	300 mg L-leucine		1 mg D-pantothenic acid
	250 mg L-lysine. HCL		(hemi-calcium salt)
	50 mg L-methionine		1 mg pyridoxal.HCl
	100 mg L-phenylalanine		0.1 mg riboflavine
	300 mg L-proline		1 mg thiamine.HCl
	400 mg L-threonine	E)	2.5 mg haemin
	50 mg L-tryptophan		
	50 mg L-tyrosine		
	100 mg L-valine		

The experimental design for the testing of promising purine compounds for antileishmanial activity was as follows. The promastigote form of the following organisms were used for testing.

- (1) Leishmania mexicana WR #227
- (2) Leishmania donovani WR #130
- (3) Leishmania braziliensis WR #424

Medium used was the defined medium of Steiger and Black lacking purine, but supplemented with 5% inactivated fetal calf serum and 0.05 mg/ml gentamicin. L. braziliensis does not grow well in this medium at 5% serum, so it was grown with 10% serum. The leishmania were initially grown in Brain Heart Infusion and when in log phase (2×10^6 cells/ml) these cells were used as an inoculum (0.5ml) and aseptically transferred to 4.5ml of Steiger and Black medium (as described) in 14.5 cm x 1.5 cm test tubes. The compound to be tested was added (at 0.1 - 500 μ M) and the cells were incubated in a slanted position at 26°C. Optical Density readings at 660nm were taken every 24 hrs. for a total of 96 hrs. Growth experiments were done in duplicate.

Compounds found to be inhibitory were retested in medium containing different purines in order to determine if the inhibitory action could be reversed with the addition of a particular purine. Purines tested for reversal of inhibitory action included: (1) Adenine (2) adenosine (3) guanine (4) guanosine (5) hypoxanthine (6) inosine.

Information gained from purine reversal experiments provides clues as to which purine enzyme or uptake process is being affected. Depending on which purine reverses we then test the analog in vitro on one of our enzyme systems. For example, if adenine reversed the inhibitory action we would test those enzyme systems which used adenine as a substrate.

Our laboratory is set up to do the following in vitro enzyme assays:

1. Adenine deaminase
2. Adenine phosphoribosyltransferase
3. Guanine deaminase
4. Hxypoxanthine-Guanine phosphoribosyltransferase
5. Nucleosidases
6. Xanthine phosphoribosyltransferase
7. Adenylate deaminase
8. Adenylosuccinate synthetase

¹⁴C-uridine into RNA, ¹⁴C-phenylalanine into protein and ¹⁴C-thymidine into DNA is determined in order to ascertain if macromolecular synthesis is being inhibited.

If we find that no salvage enzyme is inhibited but macromolecular synthesis is, we then test the following enzymes and systems:

1. DNA polymerase
2. RNA polymerase
3. incorporation of compound into RNA through use of mass spectrometry if no isotope is available.
4. inhibition of protein synthesis using a cell-free leishmanial lysate system.

Table 7

Purine analogues which have been shown to be very potent growth inhibitors of promastigotes of *L. mexicana*. The following table compares the toxicity of these compounds to some of the most promising purine analogues.

<u>Compound</u>	<u>Concentration Giving 50% Inhibition of Growth of <i>L. mexicana</i> #227 Promastigotes</u>
Sinefungin	.005
Formycin B	.1
Aphidicolin	2
4'Thioadenosine	3
Oxoformycin A	4
Deoxyaristeromycin	8
5-Deoxy-5(isobutylthio)-3-Deazaadenosine (deaza-SIBA)	20
Cordycepin	25
9-Deazainosine	40
Oxoformycin B	50
Allopurinol riboside	200

Drug Combination Tests in *L. mexicana* #227:

<u>Compound Alone</u>	<u>% Inhibition</u>	<u>In Combination With</u>	<u>% Inhibition</u>
Oxoformycin B 50 μ M	46.7	Aphidicolin (1 μ M)	52.8
BK 74731		4'thioadenosine (3 μ M)	65.2
Aphidicolin 1 μ M	26.3		
4'thioadenosine 3 μ M	46.5	Aphidicolin (1 μ M)	65
9-deazainosine 30 μ M	44.6	Aphidicolin (1 μ M)	58.8
Sinefungin .005 μ M	54.4	Aphidicolin (1 μ M)	66.1

Table 8

The most synergistic combination appeared to be 4'thioadenosine (3 μ M) and aphidicolin (1 μ M) followed closely by 9-deazainosine (30 μ M) and aphidicolin (1 μ M) and sinefungin (.005 μ M) and aphidicolin (1 μ M).

The other combinations were 20-27% below the expected combination values.

Compounds were tested to determine if they could reverse the toxicity of some of the most potent purine analogues we have tested:

<u>Purine Analog</u>	<u>Compounds Found to Reverse (100μM)</u>	<u>% Reversal</u>
4'Thioadenosine (3 μ M)	Adenosine	73
	Deoxyguanosine	46
	Thymidine	12
	Adenosine and Thymidine	76
Deoxyaristeromycin (8 μ M)	Adenosine	52
	Deoxyguanosine	31
	Thymidine	12
	Deoxyadenosine	51
Sinefungin (.005 μ M)	*	

Compounds tested in reversal experiments at 100 μ M included:

adenosine
deoxyadenosine
deoxycytidine
deoxyguanosine
L-methionine
folic acid
thymidine
thymidine and adenosine
adenosine and L-methionine

*SAM - depends when added

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